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PB-268 484

IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests for Pilot Studies

Battelle Columbus Labs, Ohio

Prepared for

Industrial Environmental Research Lab, Research Triangle Park, N C

Apr 77

PB 268 484

EPA

EPA-600/7-77-041
April 1977

IERL-RTP PROCEDURES MANUAL: LEVEL 1 ENVIRONMENTAL ASSESSMENT BIOLOGICAL TESTS FOR PILOT STUDIES

Interagency
Energy-Environment
Research and Development
Program Report

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TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-600/7-77-048	2.
3. RECIPIENT'S ACCESSION NO.	
4. TITLE AND SUBTITLE IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests For Pilot Studies	5. REPORT DATE April 1977
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) K. M. Duke, M. E. Davis, and A. J. Dennis	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Battelle-Columbus Laboratories 505 King Avenue Columbus, Ohio 43201	10. PROGRAM ELEMENT NO. EHE623
	11. CONTRACT/GRANT NO. 68-02-2138
12. SPONSORING AGENCY NAME AND ADDRESS EPA, Office of Research and Development Industrial Environmental Research Laboratory Research Triangle Park, NC 27711	13. TYPE OF REPORT AND PERIOD COVERED Task Final; 6/76-3/77
	14. SPONSORING AGENCY CODE EPA/600/13

15. SUPPLEMENTARY NOTES IERL-RTP project officer for this report is Larry D. Johnson, Mail Drop 62, 919/549-8411 Ext 2557.

16. ABSTRACT The manual gives Level 1 biological testing procedures (recommended by Industrial Environmental Research Laboratory--Research Triangle Park) for personnel experienced in conducting bioassays on samples from industrial and energy producing processes. The phased environmental assessment strategy provides a framework for industry, process, and stream priorities on the basis of a staged sampling and analysis technique. Level 1 is a screening phase that characterizes the pollutant potential of process influent and effluent streams. The manual presents the strategy of the phased approach. It also presents the basic sampling procedures and the Level 1 protocol for the biological tests used to analyze the samples. It briefly discusses possible bioassay procedures for Levels 2 and 3. The manual is a companion to 'IERL-RTP Procedures Manual: Level 1 Environmental Assessment,' EPA-600/2-76-160a, June 1976.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Air Pollution Bioassay Sampling Analysing	Air Pollution Control Stationary Sources Biological Tests Environmental Assessments	13B 08A 14B

18. DISTRIBUTION STATEMENT Unlimited	19. SECURITY CLASS (This Report) Unclassified	21. NO. OF PAGES 119
	20. SECURITY CLASS (This page) Unclassified	22. PRICE PC $4.06 / MF $0.83

EPA Form 2220-1 (9-73)

EPA-600/7-77-043
April 1977

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Program Element No. EHE623

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Prepared for

U.S. ENVIRONMENTAL PROTECTION AGENCY
Office of Research and Development
Washington, D.C. 20460

ABSTRACT

This manual gives the tentative Level 1 biological testing procedures (recommended by Industrial Environmental Research Laboratory - Research Triangle Park) for personnel experienced in conducting bioassays on samples from industrial and energy producing processes. The phased environmental assessment strategy provides a framework for prioritising feedstock and waste streams according to toxicity on the basis of a staged sampling and analysis technique. Level 1 is a screening phase that characterises the pollutant potential of process influent and effluent streams. This manual is a companion to the "IERL-RTP Procedures Manual: Level 1 Environmental Assessment", EPA-600/2-76-160a, June, 1976.

The manual presents the strategy of the phased approach. Also included are the basic sampling procedures and the Level 1 biological tests used to analyse the samples.

ACKNOWLEDGEMENT

The preparation of the biological procedures manual was conducted under the direction of Larry Johnson, Industrial Environmental Research Laboratory, Research Triangle Park, North Carolina (IERL-RTP), who was the task manager. This task was part of EPA's Environmental Assessment contract number 68-02-2138 with Battelle's Columbus Laboratory. Battelle was responsible for editing this manual with Kenneth Duke acting as the Task Order manager and Herman Mack as the program manager.

Special acknowledgement is given to the Environmental Assessment Steering Committee. This committee was composed of EPA personnel in both the Office of Energy, Minerals and Industry (OEMI) and the Office of Health and Ecological Effects (OHEE) and had the responsibility of selecting the procedures to implement the phased approach to environmental assessment. Members of the subcommittee established to define the bioassays to be used for Level 1 testing: Robert Botts, Environmental Research Laboratory (ERL) - Covallis; James Dorsey, IERL-RTP; William Horning, ERL-Duluth (Newtown); Joellen Huisingh, Health Effects Research Laboratory (HERL) - RTP; Larry Johnson, IERL-RTP; Jerry Stara, HERL-Cincinnati; Gerald Walsh, ERL-Gulf Breese; and Michael Waters, HERL-RTP.

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INTRODUCTION

This bioassay procedures manual has been prepared as a guide for pilot-studies to be conducted by the Industrial and Environmental Research Laboratory of the Environmental Protection Agency, Research Triangle Park, North Carolina. To assist in its preparation, a subgroup of the Environmental Assessment Steering Committee was formed. The subcommittee, composed of EPA experts in health and ecological effects, was given the responsibility of recommending specific bioassays. The subcommittee recommended an initial series of tests, which was reviewed by the committee as a whole, various bioassay experts within EPA, and others in industry and universities. This manual presents the agreed upon Level 1 biotests and is the result of the efforts of the Environmental Assessment Steering Committee.

It is written so that the sampling and analysis professional can plan and execute the sampling and bioassay portion of an environmental source assessment program. This manual is not intended for use by an inexperienced professional staff or by technicians. The recommended biotests for testing the toxicity and mutagenicity of feed and waste streams of industrial processes is presented. Also included is a brief summary of procedures for collecting the samples to be tested. A more detailed discussion of the sampling program is provided in the companion procedures manual, "IERL-RTP Procedures Manual: Level 1 Environmental Assessment" (Ref. 1), which also provides the procedures for chemical and physical testing of industrial process feed and waste streams.

The bioassay procedures in this manual are designed to complement the chemical and physical procedures and to be an integral part of the phased environmental assessment. They apply to Level 1. The purpose of Level 1 efforts is to obtain preliminary environmental assessment information, identify problem areas, and provide the basis for the prioritization of streams for further consideration in the overall assessment. A detailed discussion of the approach along with the criteria used for method selection is given in Chapter I.

Chapter II of this manual briefly discusses the types of Level 1 sampling activities that can be used in most industrial complexes: gas and vapor samples, gaseous streams containing particulate matter, fugitive emissions sampling, liquid and slurry sampling, and solids sampling. In

this way, the complex and difficult task of organizing the manpower and equipment necessary for successful field sampling is facilitated. For each sample type, the discussion focuses on the general problem, preparations needed for sampling, the actual sampling procedures, and packaging of samples for shipment.

Chapter III specifies the Level 1 bioassay schemes. The schemes identify the methods of analysis, anticipated output, and predicted level of effort required for implementation. The format for presenting the results of the tests is also given.

Chapter IV provides a brief discussion of a possible approach to Levels 2 and 3 biological testing.

CHAPTER I

STRATEGY AND GENERAL INFORMATION

The Industrial and Environmental Research Laboratory of the Environmental Protection Agency, Research Triangle Park, North Carolina (IERL-RTP) has developed a three-phased approach to performing an environmental source assessment--the testing of feed and waste streams associated with industrial processes in order to define control technology need. Each phase involves distinctly different sampling and analytical activities. While all three phases are briefly described in Section 1.1, this biological procedures manual focuses on the Level 1 sampling and bioassay effort. A second manual provides the chemical and physical Level 1 procedures and a detailed discussion of sampling (Ref. 1).

This manual describes for an experienced professional a set of sampling and analytical procedures for biological testing which are compatible with the information requirements of a Level 1 environmental assessment. An environmental assessment involves multimedia environmental source sampling. The sampling techniques described in Chapter II will provide an adequate sample of fugitive air and water emissions, ducted air and water emissions, liquids and slurries, and solids for the analyses described in Chapter III. Finally, Chapter IV briefly discusses Levels 2 and 3 of the phased approach to environmental source assessment.

1.1 DEFINITION OF STRATEGIES

It should be stressed that the results of Level 1 tests are not to be used for regulatory recommendations, nor are they to be used as tests of acceptability or non-acceptability. The three-phased sampling and analytical strategy was developed to focus available resources (both manpower and dollars) on emissions which have a high potential for causing measurable health or ecological effects, and to provide chemical and biological information on all sources of industrial emissions. Discussions of this philosophy, the information cost-benefits, and a summary of the application of the phased approach to sampling and analysis follow.

1.1.1 The Phased Approach

The phased approach requires three separate levels of sampling and analytical effort. The first level (1) provides preliminary environmental assessment data, (2) identifies problem areas, and (3) generates the data needed for the prioritization of energy and industrial processes, streams within a process, and components within a stream for further consideration in the overall assessment. The Level 2 sampling and analysis effort, is designed to provide additional information that will confirm and expand the information gathered in Level 1. Level 1 results serve to focus Level 2 efforts. The Level 2 results provide a more detailed characterization of biological effects of the toxic streams, define control technology needs, and may, in some cases, give the probable or exact cause of a given problem. Level 3, utilizes Level 2 or better sampling and analysis methodology in order to monitor the specific problems identified in Level 2 so that the toxic or inhibitory components in a stream can be determined exactly as a function of time and process variation for control device development. Chronic, sublethal effects are also monitored in Level 3.

To meet the environmental source assessment requirement of comprehensiveness, the phased approach provides for physical, chemical, and biological tests. Physical and chemical characterization of environmental emissions is critical to the definition of need for and design of control technology. However, the final objective of the Industrial Environmental Research Laboratory's environmental assessment is the control of industrial emissions to meet environmental or ambient goals that limit the release of substances that cause harmful biological (health and ecological) effects. Consequently, the testing of industrial feed and waste streams for biological effects is needed to complement the physical and chemical data and ensure that the assessment is comprehensive. Biological testing can provide a direct measure of toxicity and mutagenicity of substances to organisms that the other tests cannot. This is especially important when dealing with substances for which there is little available data on toxicity or when assessing complex mixtures where synergisms and antagonisms may alter the toxicity of the individual components. The use of biological tests in concert with the other tests will provide a better data base for the prioritization of streams for further study on the process of defining the need for control technology.

3.1.2 Strategy of the Phased Approach

The phased approach recognises that it is impossible to prepare for every conceivable condition on the first sampling or analysis effort. In some cases, unknown conditions and components of streams will result in unreliable information and data gaps that will require a significant percentage of the sampling or analysis effort to be repeated.

There is a possibility that many streams or even the entire installation may not be emitting hazardous substances in quantities of environmental significance. Conversely, certain streams or sites may have such problems that a control technology development program can be initiated in parallel with a Level 2 effort. If either of these situations could be determined by a simplified set of sampling and analysis techniques, considerable savings in both time and money could result.

A second possibility is that budgetary limitations may require prioritising a series of installations so that the available funds can be used in assessing only those installations most in need of control technology. Here again, a simplified sampling and analysis methodology would be advantageous to the overall environmental assessment effort.

The phased approach offers potential benefits in terms of the quality of information that is obtained for a given level of effort and in terms of the costs per unit of information. This approach has been investigated and compared to the more traditional approaches (Ref. 2) and has been found to offer the possibility of substantial savings in both time and funds required for assessment.

The three sampling and analysis levels are closely linked in the overall environmental assessment effort. Level 1 identifies the questions that must be answered by Level 2, and Level 3 monitors the problems identified in Level 2 to provide information on chronic effects and for control device design and development. The following situation is an example of this procedure.

Level 1 biological testing indicates that a small quantity of an effluent has inhibitory effects on algal growth, adverse effects on a specified percentage (EC50) of the population in a static bioassay, and gives a positive microbial mutagenicity test. Level 1 chemical testing indicates further that polycyclic organic materials (POM) might be present in significant amounts. Considering these results, Level 2 biological sampling and analysis will be

designed to determine such factors as toxic effect over a long time period, bioaccumulation at low trophic levels (primary producers and consumers), and persistance of toxicity in the receiving waters. Level 2 chemical testing will be used to further identify and quantify the POM compounds and any other significant materials as accurately as possible. This combination of biological and chemical testing can identify the exact nature of the toxic substance(s) and determine if a complex biological effect such as synergism, antagonism, or bioaccumulation is occurring. Level 3 testing will be used for long-term, continuous monitoring. Chemical testing will provide information on seasonal or feedstock variations of the previously identified toxic substance(s). Long-term biological testing will serve as an integrator of such variations. In addition, Level 3 biological testing will identify possible chronic health and ecological effects. The entire data package can then be used to design the control technology research and development program for the stream.

A detailed explanation of Level 1 sampling and analyses along with the expected outputs is given in the following sections. For Level 2 and 3, only the philosophy of the approach and some examples of the kind of tests that might be used are given. A recommended protocol for Level 2 and 3 is beyond the scope of this manual.

1.1.2.1 Definition of Level 1 Sampling and Analysis

The Level 1 sampling and analysis goal is to identify the pollution potential of a source. At the initiation of an environmental assessment, little is known about the specific sampling requirements of a source both practically and technically, and hence the emphasis is on survey tests. For this reason, no special procedure is employed in obtaining a statistically representative sample and the accuracy of the analysis is dependent on the characteristics of the sample. At this level, sampling and analysis is designed to show within broad general limits the presence or absence and the approximate levels of toxicity associated with a source. Toxic effects are further divided into health effects and ecological effects.

The results of this phase are used to establish priorities for additional testing among a series of energy and industrial sources, streams within a given source, and components within streams. Level 1 has as its

most important function the selection, in order of relative toxicity, of specific streams and components for the Level 2 effort. It delineates specific sampling, analysis, and decision-making problem areas, and directs and establishes the methodology of the Level 2 effort so that additional information needs can be satisfied.

1.1.2.2 Definition of Level 2 Sampling and Analysis

The Level 2 sampling and analysis goal is to provide definitive data required in the environmental assessment of a source. The basic questions and major problem areas to be addressed have been defined in Level 1 for maximum cost and schedule efficiency. Consequently, Level 2 sampling and analysis is characterised by obtaining statistically representative samples, expanding information on the nature of the toxicity or mutagenicity, and finally by identifying and quantifying the toxic substance(s). Level 2 analyses are the most critical of all three levels because they must provide a validation or confirmation of the results obtained in Level 1 and give a better characterisation of the potential of the sample to cause adverse environmental effects.

Level 2 thus provides sufficient detailed information concerning the problems delineated by Level 1 that control stream priorities, total environmental insult, and an initial estimate of process/control system regions of overlap can be established.

1.1.2.3 Definition of Level 3 Sampling and Analysis

Level 3 testing is very specific to the stream components being monitored, and it is not possible to define the exact tests that may be necessary. The sampling and analysis are directed towards the integration of effects over time to account for seasonal or feedstock variations. These efforts are also geared to assess the chronic health and ecological effects of the stream components.

1.2 MULTIMEDIA SAMPLING PROCEDURES

The Level 1 procedure described in this manual can be utilised to acquire process samples, effluent samples, and feed stock samples. The Level 1 environmental assessment program must, at a minimum, acquire a sample from each process feed stock stream, from each process effluent

stream, and of fugitive air/water emissions. The feed stream data are necessary to establish a baseline for comparison. The effluent stream sampling program is required to determine the mass emissions rate and the environmental insult which will result. Sampling and analytical procedures which are required for a comprehensive environmental source assessment must be multimedia in nature.

1.3.1 Classification of Streams for Sampling Purposes

The basic multimedia sampling strategy has been organized around the five general types of sampling found in industrial and energy producing processes rather than around the analytical procedures that are required on the collected samples. This facilitates the complex and difficult task of organizing the manpower and equipment necessary for successful field sampling and establishing meaningful units of cost.

The five sample types are:

(1) Gas/Vapor - These include samples from input and output process streams, process vents, and ambient air.

(2) Liquid/Slurry Streams - Liquid streams are defined as those containing less than 5 percent solids. Slurry streams are defined as those containing greater than 5 percent solids.

(3) Solids - These include a broad range of material sizes from large lumps to powders and dusts, as well as non-flowing wet pastes. Because the distinction between solids and slurries can become blurred, the reader should consult Reference 1 when in doubt.

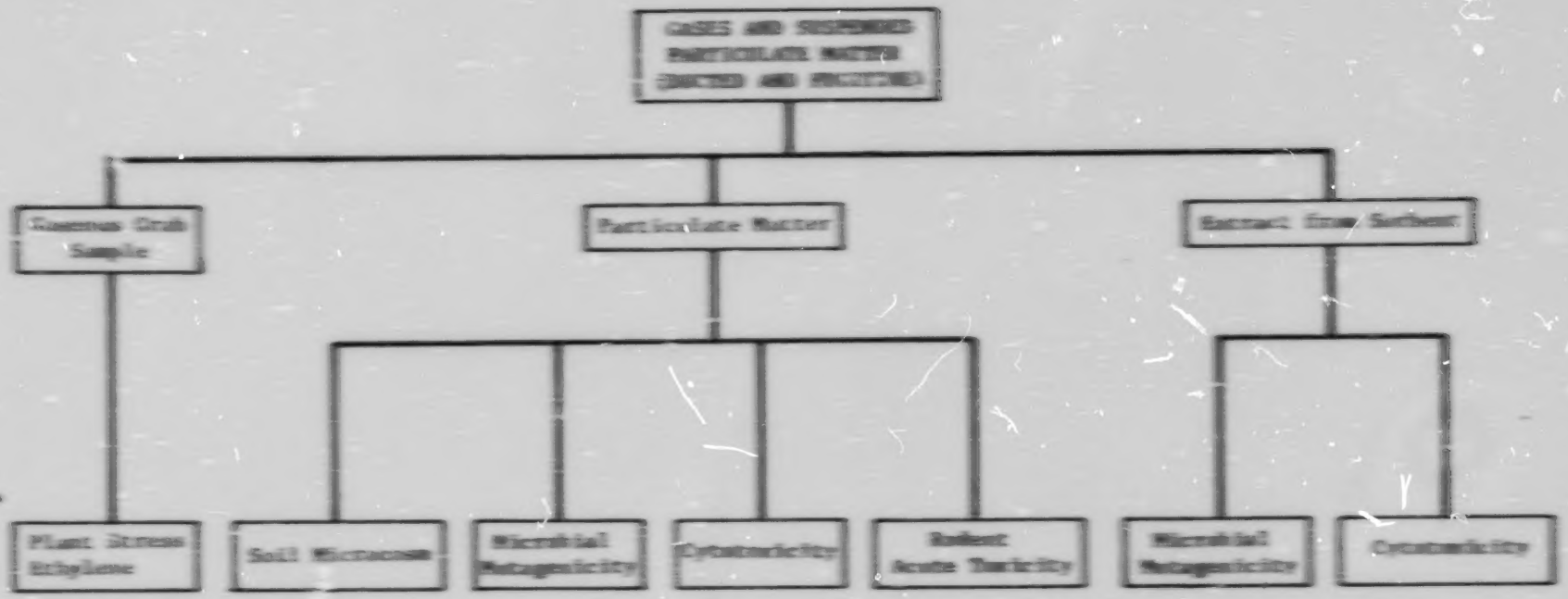
(4) Particulate or Aerosol Samples - This involves sampling in contained streams such as ducts or stacks.

(5) Fugitive Emissions - These are gaseous, particulate, or liquid emissions from the overall plant or various process units.

Flow diagrams which show the overall relationship of the samples to the Level 1 analysis scheme are presented in Figures 1 and 2.

1.3.2 Phased Approach Sampling Point Selection Criteria

The selection of sampling points in processes where phased level sampling techniques are employed relies on the concept previously stated: that Level 1 sampling is oriented towards obtaining data with relaxed accuracy requirements for determination of the pollution potential of a source, whereas Level 2 sampling is intended to acquire more accurate data necessary for a definitive environmental assessment on prioritized streams. Stream parameters such as flow rates, temperature, pressure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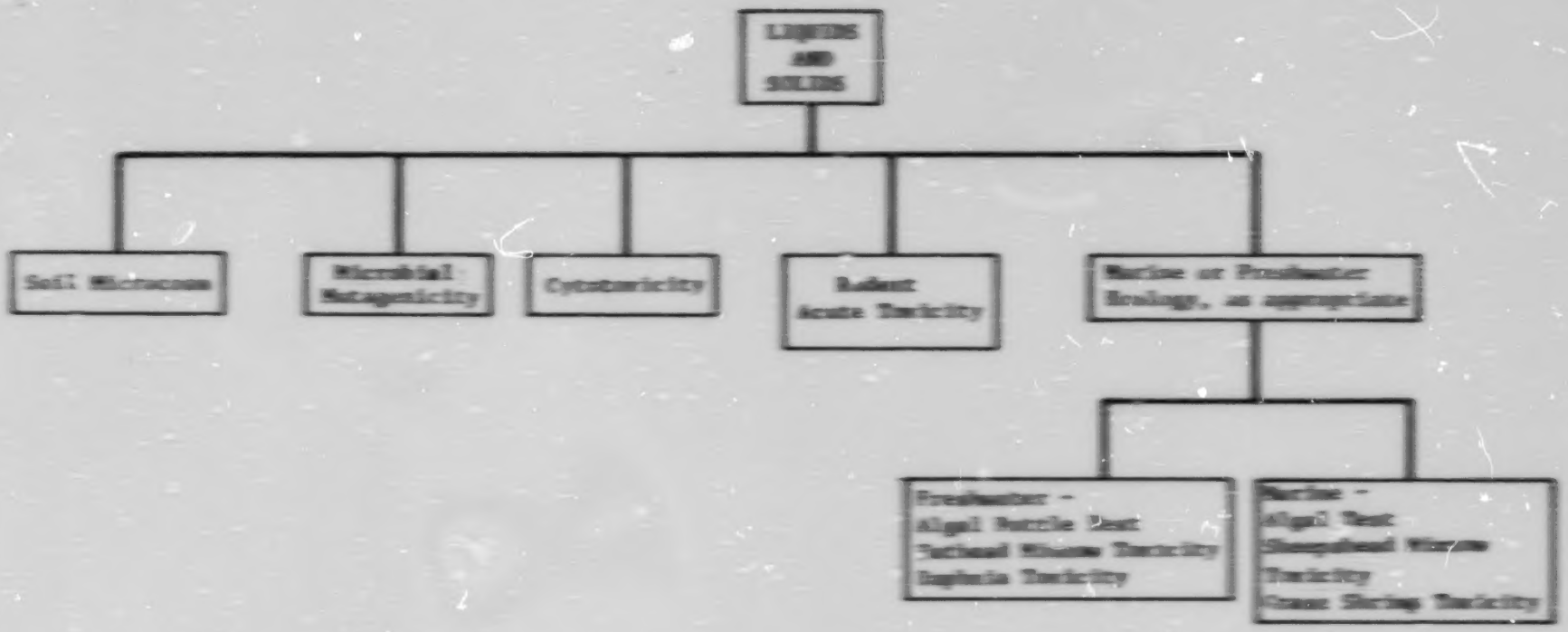

FIGURE 2. SCREENING PROTOCOL FOR LIQUID AND SOLID STREAMS

and other physical characteristics will be obtained on both levels within the objectives of a given level of sampling. The recommendations in this manual are restricted to Level 1 sampling and analysis criteria.

In most cases, Level 1 sampling methods encompass approved standard Environmental Protection Agency (EPA), American Society for Testing and Materials (ASTM), and American Planning Institute (API) techniques. Modifications are made to these techniques to adapt them to the time and cost constraints consistent with the Level 1 sampling philosophy. These modifications include: (1) reducing port selection criteria; (2) eliminating the requirements for traversing, continuous isokinetic sampling, and replicate sampling in the collection of particulate matter; and (3) using grab samples for ambient, water, and solid samples.

1.2.3 Stream Prioritization Using the Phased Approach

Industrial and energy producing processes are highly complex systems consisting of a wide variety of interrelated components. Level 1 sampling will show that many influent and effluent streams have no environmentally significant impact. These data can be used to reduce the number of samples required for Level 2 substantially, and can permit reallocation of resources. Thus, comprehensive stream prioritization based on the Level 1 sampling and analysis effort will identify streams with widely varying environmental priorities. In many cases, the Level 1 information will be sufficient to eliminate certain streams entirely from the Level 2 effort. In other cases, limited resources may require the omission of certain low priority streams.

1.3 DATA REQUIREMENTS AND PRE-TEST PLANNING (References 1-3)

The final decision to test a particular plant will depend on the results of the prioritization studies, on the preliminary selection process based on the site selection criteria of a given program, and on the data requirements of the overall program or general EPA objective.

Before the actual sampling and analysis effort is initiated, the data requirements must be established and used to help identify test requirements as well as any anticipated problems. The following paragraphs present a general summary of these requirements and planning functions which must be applied or expanded to meet the needs of the individual tests to be performed. Specific recommendations concerning data requirements associated with each of the process streams are discussed in the appropriate chapters of this manual.

1.3.1 Process Data Needs

Before traveling to a plant for a pre-test site survey, it is necessary to become familiar with the process used at the site. This involves understanding the chemistry and operational characteristics of the various unit operations as well as any pollution control processes. It is particularly important to know that detailed relevant process data are necessary for the sampling and analysis effort as well as for the overall environmental assessment in order to:

(1) Know where to look for waste streams, including fugitive emissions.

(2) Know how plant operating conditions are likely to affect waste stream flow rates and compositions.

(3) Permit design of a proper sampling program.

(4) Draw conclusions about pollutants likely to be found in waste streams.

(5) Extrapolate to conditions in other sizes of the system being assessed from thorough knowledge of the interrelationships among process variables.

(6) Develop a checklist of the requisite data including temperatures, pressures, flow rates, and variations of conditions with time for the pre-test site survey.

For any given sampling and analysis task, the data collected must be consistent with the overall Level 1 objectives. Thus, the minimum amount of data for a given stream is flow rate per unit time at a given temperature and pressure. Additional data that may be necessary are average flow per unit time, the effect of process variations on stream flow and composition, and normal variations in flow and compositions with variations in process cycling. It is expected that professional sampling and analysis personnel in conjunction with the EPA Project Officer and the Process Measurements Branch--Industrial Environmental Research Laboratory--Research Triangle Park (PMB-IERL-RTP) will select the appropriate data requirements for a given industry.

1.3.2 Pre-test Site Survey

After establishing the necessary process data needs and selecting a tentative set of sampling points, a pre-test site survey should be performed. At the test site, the survey team should meet with the plant engineer to verify the accuracy of the existing information and arrange

for the addition of any missing data. Using this information, the survey team will then proceed to select the actual sampling sites with the following criteria in mind:

(1) The sampling points should provide an adequate data base for characterizing the environmental impact of the source.

(2) When possible, each sampling point should provide a representative sample of the effluent streams.

(3) The sampling site must have a reasonably favorable working environment. The survey personnel must consider the temperature and noise level in the sampling areas, if protection from rain or strong winds exists, and whether safe scaffolding, ladders, pulleys, etc. are present.

Identification of support facilities and services is an important aspect of the site survey. Generally, the sampling team will arrive in a mobile lab unit carrying (1) sufficient electric generating capacity to operate all testing and support equipment and (2) a water tank for essential services. In an effort to minimize (1) the requests made upon the operators for support services and (2) to minimize scheduling problems, it is desirable but not mandatory that the mobile lab unit operate independently of external support facilities. Where available, electrical power and water services may be connected for auxiliary service.

The results of the pre-test site survey must be sufficiently detailed to enable complete definition of the field-test problem of sampling the correct process stream at the proper sampling location and using the appropriate methodology prior to arrival of the field-test team at the source site.

1.3.3 Pre-test Site Preparation

Since in most cases the manpower requirements for site preparation are low to moderate, a relatively low effort is assumed for site preparation. Major modifications required in extreme cases are out of the scope of this manual.

Thus, it is assumed that the erection of scaffolding and the provision of power will be a major part of site preparation; a further assumption is that the required manpower will be associated to a large extent with stack sampling, the most complex sampling procedure. Preparation of other sites is assumed to be minimal and/or part of the actual sampling procedure. The installation of special samplers, valves, fittings, etc. is considered beyond the scope of a Level 1 sampling effort.

1.4 ANALYSIS OF SAMPLES

Chapter III discusses biological analysis procedures that will provide the data requirements specified above for Level 1 environmental assessment. These discussions identify the proposed methods of analysis, the type and format of data generated, and the estimated time required to implement each analysis.

There are two categories of analysis:

(1) Health Effects Bioassays - These tests are used to detect toxic and/or mutagenic effects in bacterial and mammalian systems. These include a mutagenesis test using Salmonella (Ames test), an acute toxicity test using rodents, and a cellular toxicity test using rabbit alveolar macrophages and other mammalian cells.

(2) Ecological Effects Bioassays - These tests are used to detect toxic or inhibitory effects on the soil-litter community and on selected species of fish, macro-invertebrates, algae, and higher plants.

CHAPTER II

SAMPLING

2.1 INTRODUCTION (References 1, 3, 4)

Level 1 sampling stresses the concept of completeness by presuming that all streams leaving the process will be sampled unless empirical data equivalent to Level 1 programmatic outputs already exists. Further, Level 1 sampling is not predicated on a priori judgements as to the composition of streams. The techniques prescribed presume that whatever prior knowledge is available is at best incomplete. Predictive and extrapolation techniques employed during source assessments serve as a check on the empirical data and not as a replacement for it (Ref. 3).

Level 1 sampling programs are designed to make maximum use of existing samples and stream access sites. While some care must be exercised to ensure that the samples are not biased, the commonly applied concepts of multiple point, isokinetic, or flow proportional sampling are not rigidly adhered to. Normally, a single sample of each stream should be collected under average process operating conditions or, alternatively, under each condition of interest. These samples should be time-integrated over one or more process cycles. When a series of discreet samples results, they are combined to produce a single "average" for analysis.

This chapter discusses briefly the general methodology for obtaining gaseous, particulate, liquid, and solid feedstock and waste-stream samples for the biological analyses. Only an overview of the sampling procedures is presented. A more detailed description of procedures is found in the IERL-RTP Procedures Manual: Level 1 Environmental Assessment (Ref.1). The sampling methodology is designed to collect, prepare, and ship the samples in a manner compatible with the sample requirements for the biological tests. Table 1 characterizes the samples that will be available for the bioassays.

TABLE 1. LEVEL I SAMPLE FRACTIONS (MINIASSAY)

Source	Sample	Description	Comments
Air			
Process Gases	SASS - 2-C +SASS - 3	Solids > 1μm	May be inorganic, organic or both. Approximately 50 grams maximum
	SASS - 4	Solids 1-3μm	Same as above except 20 grams maximum
Process Fugitive Emissions	SASS - 6	Solids < 1μm	On fiberglass mat. Combine with SASS-4 if possible
	SASS - 7-C	XAD-2 extract	Organics in pentane. If solids present, may have additional solvent present. 5 grams maximum
	CYCLONE	Gas	Organic, inorganic, or both. Unfiltered sample
	MM - 1	Solids	Organic, inorganic, or both. Less than 0.5 grams
Fugitive Gases	MM - 2-C	XAD-2 extract	Same as SASS - 7-C. Less than 0.5 grams
	CYCLONE	Gas	Same as CYCLONE above
Water			
All Sources	W - 1	Extracted	Aqueous, organic, solids; unfiltered sample except for fugitive run-off
Solids			
Piles, Conveyors, Bins, etc.	S - 1	Extracted solids	Coal, ash, residues, products; organic and inorganic; unfiltered sample

3.2 GAS AND VAPOR SAMPLING

A single grab sample of each stream to be tested is sufficient for Level 1 needs. The grab sample may be taken in one of three ways depending on the pressure of the stream in question. The three grab sampler types are high pressure line, grab purge, and evacuated grab. Only the lower molecular weight organic and inorganic gaseous effluents are sampled using these procedures. The particulate content along with high molecular weight hydrocarbons are obtained via the Source Assessment Sampling System (SASS) discussed below.

For chemical analysis on-site gas chromatography, a 3-liter, glass-bulb sample container is used. For biological testing, a much larger quantity of gas is required and the sample must be shipped for analysis; therefore, the sample must be collected in a larger container. Various containers can be used including large Teflon or Tedlar bags, and rigid glass or stainless steel containers.

3.3 SAMPLING OF GASEOUS STREAMS CONTAINING PARTICULATE MATTER

Streams, vents, and effluents containing particulate matter are sampled using the Source Assessment Sampling System* (SASS) developed by the Industrial Environmental Research Laboratory of the Environmental Protection Agency, Research Triangle Park (IERL-RTP). (Sampling of fugitive gaseous emissions containing particulate matter is accomplished using the high-volume sampler discussed below.) The SASS sampling train consists of a stainless steel probe which enters an oven module containing three cyclones and a filter. Size fractionation is accomplished in the series to provide large quantities of particulate matter size-classified into three ranges: (1) >10um, (2) 3um to 10um, and (3) 1um to 3um. Particulates < 1um are collected with the filter. Volatile organic material is collected in a XAD-2 sorbent trap, a porous polymer resin with the capability of adsorbing a broad range of organic species. Some trapping of volatile inorganic species is also anticipated as a result of simple impaction. After all fractions have been removed by the SASS train; a grab sample of the gaseous stream can be taken as described in the previous section.

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*Manufactured by Aerotherm Corporation, 485 Clyde Avenue, Mountain View California 94042, Telephone (415) 964-3300.

Whether or not quantities sufficient for analysis have been acquired cannot be determined until subsequent gravimetric analyses have been performed; therefore, some sampling guidelines should be followed. At least one process cycle and 30 standard m^3 of the process effluent are to be sampled. If the process is not cyclic in nature, the 30 standard m^3 must still be satisfied over a period of time conducive to obtaining a sample representative of process conditions. A sampling duration of 8 hours generally satisfies this requirement and provides enough material for chemical analysis. A longer period may be necessary to obtain adequate samples for biological analysis.

At the conclusion of the sampling run, the train is disassembled and transported to the mobile lab unit or prepared work area. The three cyclones and the filter chamber must each be tapped and brushed to remove all contents on the walls. Liquids should not be used in removing particulates for biological analysis. The 10μm and 3μm cyclone particulates are combined as are the 1μm and filter chamber particulates. The filter should also be tapped to remove particulate matter, which is added to the smaller fraction. Since it may be very difficult to remove all particulates, this fraction may be tested separately. The filter should not be brushed or scraped as this process leaves glass fibers in the sample. For shipping, the two particulate fractions are each transferred to a tared nalgene container. The filter is also shipped in a tared petri dish. The XAD-2 cartridge is removed from its container and placed into a widemouth, amber-glass jar. All containers are shipped to the environmental assessment laboratory for further preparation, splitting, and shipping to appropriate labs for biotesting.

2.4 FUGITIVE EMISSIONS SAMPLING

Fugitive emissions are those air and water pollutants generated by any activity at an industrial site that are transmitted from their source directly into the ambient air or receiving surface and ground waters without first passing through a stack, duct, pipe, or channel designed to direct or control their flow. Airborne emissions consisting of particulate matter and gaseous pollutants may be generated by sources

enclosed in buildings and transmitted to the atmosphere through structural openings or vents, or generated by sources in open areas and transmitted directly into the atmosphere. Waterborne fugitive emissions, which consist primarily of suspended and dissolved solids, may be generated by process leaks and spills, runoff from a wide variety of material storage piles, and fallout from emissions initially airborne. They are transmitted to surface waters by runoff and to ground waters by infiltration.

2.4.1 Airborne Fugitive Emissions

Airborne fugitive emissions may be of three types each requiring different sampling procedures: (1) a site source in which no specific source of emissions can be identified, (2) a specific source which generates a highly diffuse cloud over an extensive area, or (3) a specific source which generates an emission which could be generally classified as a plume. In case 1, samples must be taken at both an upwind and a downwind location. Particulates are sampled with a high volume sampler equipped with a 3.5µm filter and a XAD-2 cartridge. Gases are sampled with an evacuated grab sampler. In case 2, samples are taken at a downwind location only. The high volume sampler is used to collect the particulate fraction and an evacuated grab sampler is used to collect a gaseous sample. In case 3, the plume is sampled using the SASS train described in the previous section and an evacuated grab sampler.

After sampling, particulates are tapped and brushed from the 3.5µm fractionating head and tapped from the filter into a tared nalgene container for shipping. The XAD-2 cartridges are placed in amber-glass jars.

2.4.2 Waterborne Fugitive Emissions

Waterborne fugitive emissions are sampled using plug collectors.* The plugs are driven into the ground at selected locations where runoff will occur. Water runoff samples are similar to airborne fugitive emissions in that both specific source and site source samples are obtainable. Collectors placed for general plant runoff are analogous to site source upwind-downwind samples, and collectors placed for specific

* Plugs are designed and built by Kahl Scientific Instrument Corp., P.O. Box 1166, El Cajon, California 92022.

problem areas are analagous to specific source downwind samples. Runoff samples from specific sources are combined in order to perform a single analysis to characterize a potential problem area. Waterborne fugitive emission samples are handled and analysed as are other liquid samples, as described in the following section.

2.5 LIQUID AND SLURRY SAMPLING

Many diverse influent and effluent streams exist in liquid or slurry form. Three sampling methods are used for sampling liquid streams: heat exchange, tap sampling, and dipper sampling. The heat exchange system is used for high-temperature lines and involves the use of a water-cooled condenser system. The condensate from the stream is collected in a reservoir for later analysis. Tap sampling is used to sample both moving streams (lines) and non-moving streams (tanks or drums). It involves taking a sample at a tap from a line or tank wall. Dipper sampling is applicable to sluices or open discharge streams of thick slurry or stratified composition. The dipper is made with a flared bowl, coated with teflon, and attached handle. It is inserted into the free-flowing stream so that a sample is collected from the full cross-section of the stream.

Crude liquid effluent containing aqueous, organic, and solid material should be tested for health and ecological effects. The material should generally be transported in tightly sealed, amber-glass containers. Aqueous samples may be shipped in high-density polyethylene containers. All samples should be shipped and stored at 4 C and no stabilizers should be added to samples to be used in bioassays. In addition, special care should be taken not to agitate the samples or to open the containers before analysis.

2.6 SOLID SAMPLING

Solid samples range in size from large lumps to fine powders and dusts and in consistency from anhydrous solids to thick, nonflowing pastes. Level 1 solid sampling procedures use manual grab sampling techniques such as shovel or grab sampling, boring techniques including pipe or thief sampling, and auger sampling.

Samples should be stored in air-tight, high-density polyethylene containers until ready for analysis. Large samples should be placed in metal containers lined with polyethylene bags.

CHAPTER III

LEVEL 1 BIOASSAY TECHNIQUES

3.1 INTRODUCTION

Level 1 environmental source sampling procedures provide a set of samples which represent the "average" composition of solid, liquid, and gaseous feed or waste streams of industrial processes. Each fraction or stream will be evaluated with survey techniques to define its basic physical, chemical, and biological characteristics. The survey methods or tests selected are compatible with a very broad spectrum of materials and have sufficient sensitivity to ensure a high probability of detecting potential environmental problems. The methods and instrumentation have been kept as simple as possible to minimize cost but still provide the information required by Level 1 objectives. Each individual piece of data should add a relevant point to the overall evaluation. Conversely, since the information from a given test is limited, all the tests must be accomplished to permit a valid assessment of the sample. This is particularly true for the bioassays because physical and chemical information alone cannot provide a reliable measure of potential biological response. In addition, only bioassays can detect complex biological effects such as synergism and antagonism. On the other hand, bioassays cannot identify the cause of toxicity or mutagenicity or suggest means of controlling it. Thus, physical, chemical, and biological analyses must be used to complement one another at all three levels of the phased approach to environmental assessment.

Level 1 biological testing is limited to whole sample testing which is consistent with the survey nature of this level. The testing of fractionated samples or specific components of a given sample involves a degree of specificity appropriate to Levels 2 and 3 testing. This chapter includes a brief description of how the various samples should be handled in preparation for biological analysis as well as detailed descriptions of the bioassays recommended for Level 1.

The bioassays include both assessments of health and ecological effects. Health effects tests estimate the potential mutagenicity, potential or presumptive carcinogenicity, and potential toxicity of the samples to mammalian organisms. The ecological effects tests focus on the potential toxicity of the samples to vertebrates (fish), invertebrates, and plants in freshwater, marine, and terrestrial ecosystems. The species chosen for the tests have been used for many documented environmental assessments in most cases. A total of nine tests comprise Level 1 testing (there are two tests - marine and freshwater - for the algal and static bioassays). Table 2 shows which bioassays are to be used with each sample type and constitutes the minimal bioassay protocol. The approximate time and amount of sample required for each of the tests is shown in Table 3.

The minimal test matrix (see Table 2) shows several tests to be used as tests of secondary priority for various sample types. These tests can give useful Level 1 data and should be used if time, money, and the amount of sample available for testing permit. This is an example of the flexibility that is possible in implementing the Level 1 bioassays. The minimal test procedures should be performed first. If conditions permit, additional testing may be performed at this Level. This testing would serve to clarify confusing or inconclusive results or provide additional data that would permit a better prioritization of the various influent and effluent streams. Such additional testing, if done, should be designed to meet the objective of Level 1.

Following the bioassay descriptions is a brief section on the suggested format for reporting results of the bioassays. Results of all Level 1 bioassays plus information from chemical and physical Level 1 testing will permit the identification and relative ranking of the feedstock or waste streams so that Level 2 testing can be initiated on the most environmentally significant streams with the testing of lower priority streams to follow.

3.2 SAMPLE HANDLING

3.2.1 Introduction

Due to the variation in samples which could be received for analysis, it is impossible to provide explicit instructions on how every sample should be handled and administered to the test organisms. This ultimately

TABLE 2. LEVEL I [illegible] TEST MATRIX

Sample Type	Health Effects Tests			Ecology Effects Tests		
Water and Liquids	Microbial Mutagenesis	Rodent Acute Toxicity	Cytotoxicity	Algal Bioassay	Static Bioassays	Soil Microcosm
Solids-(Aqueous Extract Feed, Product, Waste	Microbial Mutagenesis	Rodent Acute Toxicity	Cytotoxicity	Algal Bioassay	Static Bioassays	Soil Microcosm
Gases-(Grab Sample)	(Microbial Mutagenesis)[a]		(Cytotoxicity)			Plant Stress Ethylene
Particulates	Microbial Mutagenesis	(Rodent Acute Toxicity)	Cytotoxicity			Soil Microcosm
Sorbent-(Extract)	Microbial Mutagenesis		Cytotoxicity			

(a) (Recommended Test of Secondary Priority).

TABLE 3. LEVEL I HEALTH AND ECOLOGICAL TESTS REQUIREMENTS

Test System/Level	Approximate Time Required (including analysis)	Sample
Microbial mutagenesis (Ames)	2-3 days (3 wks)	1 gram/30 ml
Cytotoxicity (mammalian cells)	2-4 days (3 wks)	1/2 gram/30 ml
Range finding toxicity (rats)	14 days (4 wks)	100 grams, 1 L
Freshwater static bioassay	4 days (1 wk)	200 L
Algal bottle assay (Freshwater)	10-14 days (3 wks)	
Marine static bioassay	4 days (2 wks)	90 L
Algal Assay (Marine)	14 days (3 wks)	
Plant stress ethylene	28 hrs (1 wk)	1360 L
Soil-litter microcosm	40 days (8 wks)	1 gram/1 ml

must be left to the discretion of the professional investigator. Some guidelines are presented below and in Figure 3. Results of chemical or physical tests done on-site and information from previously completed biological tests may also be of assistance in making such decisions as how much effluent to use and how to administer it.

3.3.2 Gaseous and Particulate Samples

Gas samples are to be tested by the plant stress ethylene test. Gas containers obtained during sampling can be connected directly to an air-handling system on the greenhouse exposure chambers to make the various dilutions necessary, or the dilutions can be pre-mixed in a second container and from this added to the exposure chambers.

Particulates can be incorporated into the bioassays as solid particles or aqueous suspensions. In general, only respirable (< 5 μm) particles should be tested as solid particles in the microbial mutagenesis or cellular toxicity tests. Larger particulates hinder growth by inhibiting adherence of cells to the flask or by other physical means and cannot be ingested by cells. The RAM test is an exception and can incorporate the large particulate fraction as solids. Particulates of both fraction sizes (respirable and non-respirable) can be administered to rodents in a variety of ways including intubation, respiration, and skin painting depending on the health problem expected to result from exposure to particulates. For the soil microcosm test, solid particulates can be added to the surface of the soil cores.

The XAD-2 sorbent is homogenized and extracted with pentane. The pentane extract must then undergo solvent exchange with, preferably, dimethyl sulfoxide (DMSO) or acetone before incorporation into the microbial mutagenesis or cellular toxicity tests as a liquid. Depending on the nature of the original sample, solvent exchange can be accomplished by several ways. An equal volume of DMSO can be added to the pentane extract and heated or evaporated at room temperature to remove the pentane, or the pentane can be removed first and the residue resuspended in DMSO.

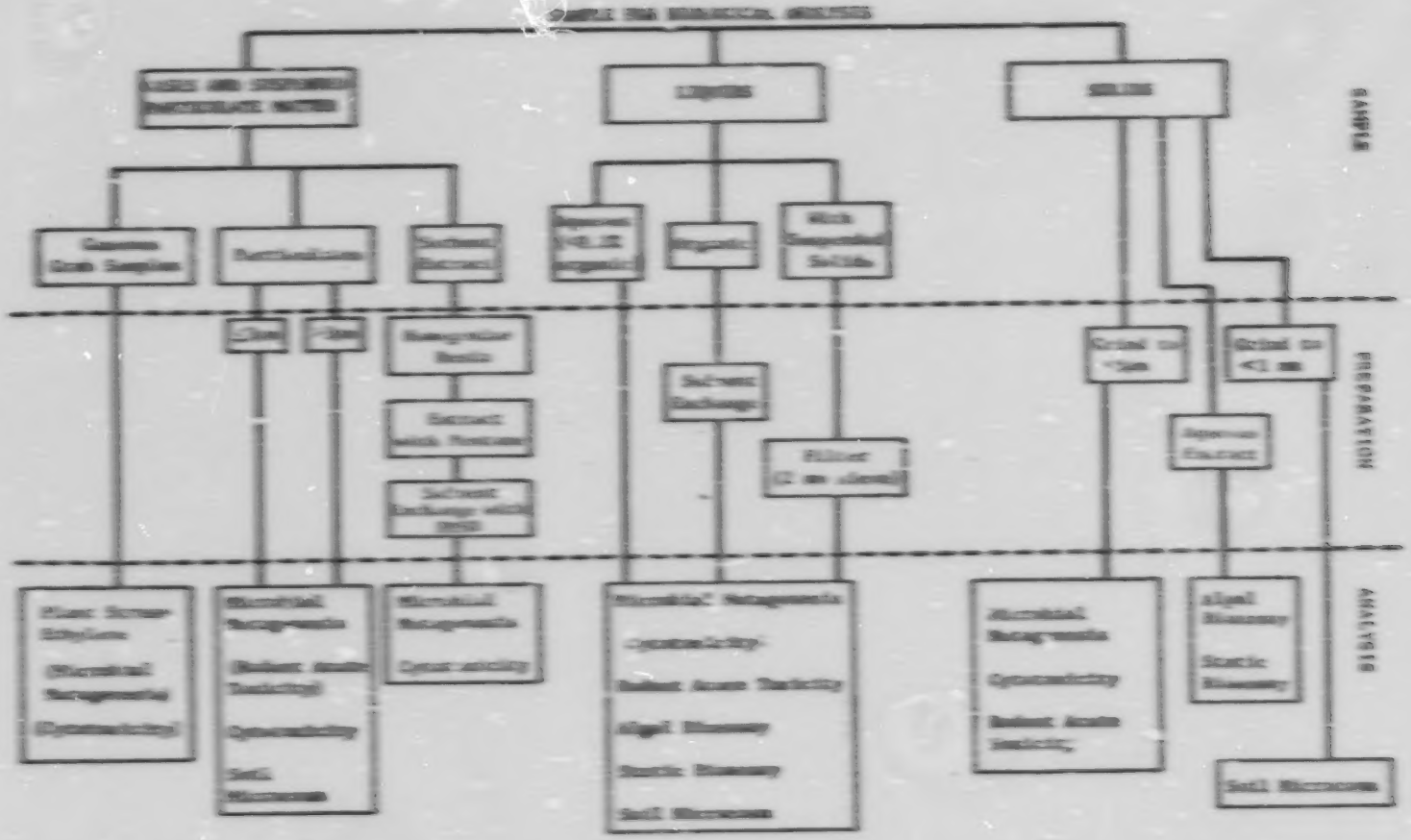

FIGURE 1.

3.3.3 Liquid Samples

Generally, liquid containing < 0.2 percent organic solvents can be added directly to the microbial mutagenesis test. If larger quantities of various organic solvents are present, and if effects due to dissolved substances are to be distinguished from those due to the solvent, solvent exchange must be performed. Final concentrations in the test medium of 0.5 percent DMSO or 0.01 percent acetone can be tolerated.

Liquids can be administered to rats by such methods as skin painting, inhalation, ingestion, and injection. Greater concentrations of organic materials can generally be tolerated with the skin painting technique.

For the ecological tests, samples should be added to the dilution water without the use of solvents or additives, except water if necessary. If additives are necessary, they should be kept to a minimum as they may affect the pH of the test solutions. Organic solvents used to prepare the sample should also be kept to a minimum. Concentration of solvent in any test solution in the algal and static bioassays must not exceed 0.5 ml/l. Temperature and salt content (if salt-water assay) of the effluent may have to be adjusted before starting the test. Other adjustments may be necessary depending on the results of on-site chemical tests. For instance, oxygen may have to be bubbled through the test solution, but agitation of the solution should be avoided. Samples that contain suspended solid material can be filtered through a sieve as small as 3 mm.

Addition of organic material or highly acidic or basic additives to the soil microcosm test should be minimized; however, no strict guidelines are available at the present.

3.3.4 Solid Samples

Solid samples should be finely ground (< 5 μm particles) and incorporated as are particulates in the microbial mutagenesis, cytotoxicity, and rodent acute toxicity bioassays, solid samples should be ground to < 1 mm particles for the soil microcosm bioassay. Aqueous extracts of solid samples should be added to the algal and static bioassays. Aqueous extracts are made by vigorously shaking or stirring 1 part solid sample and 4 parts water for 30 minutes followed by 1 hour of settling and filtration or centrifugation of the filtrate as appropriate.

3.2.9 Range-finding and Definitive Tests

For all the following bioteets, it is usually desirable to conduct a range-finding or screening test to determine the concentrations that should be used in the definitive test, unless the approximate toxicity of an effluent is already known or unless the sample size is limiting. This preliminary testing can save both time and money. Range-finding tests should be conducted using three to five widely spaced effluent concentrations (covering the entire range of 0 to 100 percent effluent, if possible). Conditions for the range-finding and definitive tests should be kept as similar as possible; the greater the similarity between the two tests, the more useful the results of the range-finding tests will be.

3.3 HEALTH EFFECTS TESTS

3.3.1 Salmonella/Microsome Mutagenesis Assay (Ames') (Reference 5)

The Ames assay is based on the property of selected Salmonella typhimurium mutants to revert from a histidine requiring state to prototrophy due to exposure to various classes of mutagens. The test can detect to nanogram quantities of mutagens and has been adapted to mimic some mammalian metabolic processes by the addition of a mammalian liver 9,000 G microsomal fraction (S-9). The test will be used as a primary screen to determine the mutagenic activity of complex mixtures or component fractions. It has recently been demonstrated that most carcinogens act as mutagens. In extensive testing, the Ames' assay has demonstrated 90 percent accuracy in detecting known carcinogens as mutagens. Certain known carcinogens are negative in the test (e.g., asbestos, metals) or weakly positive. False positives are also known which are mutagenic in the Ames' system but which have been found to be noncarcinogenic in mammals. Continued improvement of the present bacterial strains, addition of new strains, and re-evaluation of the conventional animal carcinogenesis data should reduce this level of error in the near future. The following is intended as a general description of the test; for detailed protocol see the published method. (Reference 5)

3.3.1.1 Method Description

Materials. The species of bacteria to be used are the Salmonella typhimurium tester strains developed by Dr. Bruce Ames. Specifically, these are TA-1535, TA-1537, TA-1538, TA-98, and TA-100. The tester strains

are histidine deficient variants and are used to detect frameshift reverse mutations (TA-1537, -98, and -1538) or base pair substitutions (TA-1535 and -100) as indicated by reversion to prototrophy. For general screening, strain TA-1538 may be omitted.

Liver Microsome Preparations. The activation system for mutagenesis screening consists of Aroclor 1254 induced S-9 fraction derived from rat livers. Rats should be males (Sprague-Dawley) about 200 g each. Induction is accomplished by a single intraperitoneal injection of Aroclor 1254 (diluted in corn oil to 200 mg/ml) into each rat five days before sacrifice at a dosage of 0.5 mg/g of body weight. All rats are deprived of food (not water) 12 hours before sacrifice. The rats are then stunned by a blow on the head and decapitated. The following steps are carried out at 4 C using cold sterile solutions and glassware.

The livers (10-15 g) are aseptically removed from the rats and placed into a cold preweighed beaker containing 10-15 ml of 0.15M KCl. After the livers are washed and weighed in this beaker they are removed with forceps to a second beaker containing 3 ml of the KCl solution per gram of wet liver weight. The livers are then minced with sterile scissors, transferred to a chilled glass homogenizing tube, and homogenized in an ice bath by passing a low speed motor driven pestle through the livers a maximum of three times. The chilled homogenates are then placed into centrifuge tubes and centrifuged for 10 minutes at 9000 G at 4 C. The resulting supernatant is decanted, transferred in 2-ml amounts to small storage tubes, quickly frozen in dry ice, and stored at -80 C in a low temperature freezer. This supernatant is known as the S-9 fraction. Sufficient S-9 for use each day is thawed at room temperature and kept on ice before and during use. The extent of bacterial contamination of the S-9 fraction should be determined. The S-9 mix may be filter sterilized (0.45 um porosity filter) if required.

Metabolic Activation Mixture. The S-9 microsomal mix is prepared according to the recommendations of Ames. The mix contains per ml: S-9 (0.04-0.1 ml), $MgCl_2$ (8 umoles), glucose-6-phosphate (5 umoles), nicotinamide adenine dinucleotide phosphate (NADP) (4 umoles), and sodium phosphate, pH 7.4 (100 umoles). Stock solutions of NADP (0.1 M) and glucose-6-phosphate (1.0M) are prepared with sterile water, in appropriate amounts, and maintained

at -20 C. The stock salt solution (0.4M $MgCl_2$ and 1.6% KCl) and phosphate buffer (0.2M pH, 7.4) are prepared, autoclaved, and refrigerated. The S-9 mix is prepared fresh each day and is maintained on ice before and during use. The activity of the S-9 preparation should be standardized by measurement of its aryl hydrocarbon hydroxylase activity and by determination of its total protein content.

Bacteriological Media. The minimal-glucose agar medium for histidine requiring strains used in mutagenesis assays is a 1.5 percent Bacto-Difco agar in Vogel-Bonner Medium E with 2 percent glucose. Top agar (0.6 percent Difco agar, 0.5 percent NaCl) contains a trace of histidine and biotin to permit the bacteria to undergo several divisions. The top agar medium used in the (optional) toxicity assays is the same medium fortified with 30 mg/ml of histidine. Fewer bacteria (about 10^2 cells/plate) are used in the toxicity test.

Solvents and Positive Control Chemicals. The solvent for all stock control chemicals is a spectrophotometric grade dimethyl sulfoxide. The following compounds are examples of compounds used for positive control assays. (Ref. 3) Others may be substituted.

Indicator Strain	Nonactivation Assays	Activation Assays
TA 1535	MNNG (N-Methyl-N'-nitro-N-nitrosoguanidine)	2-anthramine
TA 1537	9-aminoacridine*	2-anthramine
TA 98	Daunomycin	2-anthramine
TA 100	MMS (Methyl methanesulfonate)	2-anthramine

*9-aminoacridine is dissolved in ethanol.

Methods. Each sample should undergo a mutagenesis test, an optional toxicity test, a positive control test, and a sterility control test. The plate incorporation test is recommended for routine use. The cells used for the toxicity and mutagenesis determinations are derived simultaneously from the same bacterial population. Viability in the toxicity assay will be used to adjust counts of mutant colonies by the estimated proportion of the surviving population.

In the plate incorporation assay the sample under investigation is added directly to the molten top agar and is poured onto the plate along with the indicator test organism and the liver S-9 activation system.

It is recommended that the plate incorporation test be performed (preferably in duplicate) initially at 0.01, 0.1, 1 and 10 mg/plate, and that repeat studies be performed over a narrower concentration range showing positive results in the initial test (up to a maximum concentration of 30 mg/plate).

The test compound is evaluated with all tester strains in the presence and absence of the liver S-9 activation system. Liquid samples are assayed similarly with the maximum sample volume being about one ml. Controls are included at all times and consist of a control for the spontaneous reversion rate for each tester strain where the mutagen is omitted, a sterility check of the mutagen solution, and a positive control consisting of compounds which both do and do not require metabolic activation.

The number of spontaneous reversions/plate should be within the following limits or those specified in Reference 5.

Strain	Acceptable Spontaneous Revertants/Plate
TA-1535	30 ± 10
TA-1537	15 ± 10
TA-98	50 ± 25
TA-100	150 ± 75

A sample will be considered negative if the number of induced revertants obtained as compared to the spontaneous revertants is less than two-fold.

Compounds which indicate positive reversions (at least a two-fold increase in reversion rate) will be examined by preparing dose response curves with the tester strain(s) indicating high reversion rates. The dose response curve will be performed in duplicate at five concentrations of the sample found to be mutagenic. A two-fold increase in reversion rate with evidence of increasing reversion with increasing sample concentration shall constitute a positive test. In addition, the dose response curve should be reproducible.

Sterility Controls. Each bioassay includes a sterility control check for each test component. These include the S-9 mix, the compound under test, and the solvent vehicle.

Phenotype Monitoring. After the number of colonies on each plate is counted, occasional samples (for example, 10) of the revertant colonies are picked from each plate exhibiting a positive response. These colonies are restreaked onto minimal agar to confirm if they are revertants or potential phenocopies.

3.3.1.2 Results

The plate incorporation mutagenesis/toxicity test provides an indication of both mutagenicity and toxicity of the sample. Toxicity is evidenced by a reduction in the number of colonies (viability) of bacteria. Counts of mutant colonies in the plate incorporation mutagenesis test are adjusted by multiplying average counts by 1/viability. Relative mutagenic activity is derived from the adjusted average colony counts by representing the control plate count (vehicle only) as 1.00 and then converting proportionately the adjusted average counts from the plates which contain test sample.

3.3.2 Cytotoxicity Assays (References 6-11)

Cytotoxicity assays employ mammalian cells in culture to measure quantitatively cellular metabolic impairment and death resulting from exposure in vitro to soluble and particulate toxicants. Mammalian cells derived from various tissues and organs can be maintained as short term primary cultures or in some cases, as continuous cell strains or lines. Primary cell cultures exhibit many of the metabolic and functional attributes of the original tissues, some of which may be lost with prolonged passage in culture. Both types of systems have been employed effectively in cytotoxicity screening of, for example, pharmaceuticals (especially antibiotics and anti-tumor agents), implantable medical polymers and mineral crystals and fibers. More recently, such systems have been applied in evaluating the relative cellular toxicity of hazardous metallic salts (Ref. 8) and industrial air particulates (Ref. 7). As compared to conventional whole animal tests for acute toxicity, cytotoxicity assays are more rapid, less costly and require significantly less sample. The tests can provide useful information about the relative cellular toxicity of unknown samples. However, it should be understood that because the assays employ isolated cells and not intact animals, they can provide only preliminary and imprecise information about the ultimate health hazards of toxic chemicals.

The cytotoxicity assays, to be evaluated as part of Level 1 analysis, employ primary cultures of rabbit alveolar (lung) macrophages (RAM) and maintenance cultures of strain WI-38 human lung fibroblasts. The alveolar macrophage constitutes an essential first line of pulmonary defense by virtue of its ability to engulf and remove particulate materials which are deposited in the deep lung. It is appropriate therefore that this cell type be used to define the acute cellular toxicity of airborne particulates and associated chemicals. It has been possible to develop a cytotoxicity screening system employing these cells to "rank" the toxicity of a series of industrial particulates collected on a cyclone sampling train similar to the SASS train (Ref. 7). The strain WI-38 human lung fibroblasts are perhaps the best characterized diploid human cell available for cytotoxicity screening. These cells exhibit the major pathways of DNA, RNA and protein synthesis common to all dividing cells and can be shown to possess a number of inducible enzyme systems.

For Level 1 assessment these two cytotoxicity assays will be performed where possible on all solid and liquid effluents. In some cases, a clonal toxicity assay will be employed for comparative purposes (utilizing an appropriate cell type, e.g., CHO, L-929 cells).

3.3.2.1 Rabbit Alveolar Macrophage (RAM) Assay

Method Description. Male and female New Zealand white rabbits are housed individually and fed antibiotic-free Purina Rabbit Chow and water ad libitum. Clinically healthy rabbits weighing 1.5-2.5 kg are sacrificed by injection of sodium pentobarbital (150 mg) into the marginal ear vein. The animals are draped and the site of incision for tracheostomy liberally irrigated with 70 percent ethanol. Lung lavage *in situ* is carried out according to the procedure of Coffin et al., (Ref. 8) using prewarmed (37 C) sterile 0.95 percent saline. The first 30 ml vol instilled into the lungs is allowed to remain for 15 minutes; five subsequent instillations of 30 ml each are withdrawn immediately. Lavage fluid found to contain blood or mucous is discarded. The cellular composition of the pooled lavage fluid is determined from Giemsa stained smears and is routinely 99 percent alveolar macrophages, 2-3 percent polymorphonuclear leukocytes, and 2 percent lymphocytes.

The cells are washed once by centrifugation at 169 G for 15 minutes at 25 C and resuspended in prewarmed (37 C) tissue culture Medium 199 in Hanks' balanced salt solution. Supplements added to the medium include heat-inactivated fetal bovine serum (10 percent), penicillin (100 units/ml), streptomycin (100 ug/ml), and kanamycin (100 ug/ml). (Biologicals available from Gibco, Grand Island, NY.) Cells are counted by means of a hemocytometer or automatic cell counting device and diluted to approximately 1 x 10^6 cells per ml with supplemented medium. One ml of the cell suspension is added to each well of 100 x 100 mm 4-piece cluster dishes (Falcon Plastics) containing the effluent sample, and sufficient medium is added to bring the total volume per well to 2.0 ml. The cultures are incubated, with rocking, for 20 hours at 37 C in a humidified atmosphere containing 5 percent CO_2. At the end of this incubation period, the cells are trypsinized and cell counts, cell viability, total protein, and ATP determinations are performed on cells obtained from each well.

Viability determinations. Viability determinations are performed as follows:

The culture medium is poured off and retained separately in a culture tube. Cells are dissociated by using 0.25 percent trypsin in Gibco solution A. The suspended cells are recombined with the original culture medium and chilled. Appropriate dilutions, usually 4-fold, are made by using cold 0.85 percent saline to yield a suspension of no more than 2 x 10^5 cells/ml. Trypan blue, freshly diluted with 0.85 percent saline to a final concentration of 0.01 percent is added to an equal volume of cell suspension for determination of cell viability. Simultaneous determinations of cell viability and cell numbers per milliliter of cell suspension are performed using a hemocytometer or Cytograf (Biophysics Systems, Mohapac, NY). Viability is expressed as a percentage. The viability of cells from control cultures is routinely 95 percent or greater. Cell numbers are expressed as a percentage of the numbers of cells in control cultures. Viability determinations are multiplied by total cell numbers as a fraction of control cell numbers to yield the viability index, or net number of viable cells as a percent of control.

$$\text{Viability index} = \text{Viability (\%)} \times \frac{\text{No. cells expt}}{\text{No. cells control}}$$

Protein Determinations. Total protein may be used in the place of cell number. For determination of total culture protein, cells washed twice with 0.85 percent saline are lysed in 1.0 percent sodium deoxycholate (Schwarz-Mann, Orangeburg, NY) and 0.1 ml aliquots are assayed according to the method of Lowry et al. (Ref. 9) by using a bovine serum albumin standard (Nutritional Biochemicals Corp., Columbus, Ohio).

Adenosine Triphosphate (ATP) Determinations. ATP is determined according to a procedure supplied with the DuPont model 760 Luminescence Biometer. Dimethyl sulfoxide (0.4 ml) is used to extract ATP from a 0.1 ml aliquot of trypsinized cell suspension containing 0.3-0.4 x 10^6 cells. After 2 min at room temperature, 5.0 ml of cold 0.01 M morpholinopropane sulfonic acid (MOPS) at pH 7.4 is added to buffer the extracted sample. The tube containing the buffered sample is then placed in an ice bath. Aliquots of 10 µl are injected into the luminescence meter's reaction cuvette containing 0.7 mM luciferin (crystalline), 100 units luciferase (purified and stabilized)*, and 0.01M magnesium sulfate in a total volume of 100 µl of 0.02M MOPS buffer, pH 7.4 at 25 C. Light emitted from the reaction cuvette is measured photomultrically in the luminescence meter and proportional to the ATP concentration of the sample. ATP values are expressed per 10^6 cells and as a percent of the control cells.

Measurement of Phagocytic Activity. Phagocytic activity is measured by addition of 1.1 µm polystyrene latex particles (Dow Diagnostics, Indianapolis, Indiana) to alveolar macrophages cultured in Lab-Tek (Miles Laboratories, Inc. Naperville, Ill.) four-chamber microslides (approximately 25 particles/cell in 1 ml of supplemented medium). Preparation and maintenance conditions were as previously described. One hour after the addition of latex particles, the slides are drained, air-dried, and exposed for 3 min to concentrated Wright stain. The slides are then exposed for an additional 5-6 min with a 1:1 aqueous dilution of Wright's stain. After air drying, the slides are placed in xylene for 1 hr to dissolve extracellular particles according to the procedure of Gardner et al. (Ref. 10). Following

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*Unit (1) luciferase = $\frac{\text{response to 1.64 pmole ATP}}{\text{response to 20 µCi } {}^{14}C \text{ calibrated light source}}$

an additional drying step, the slides were mounted in permount. Phagocytic activity is determined under oil immersion by scoring a minimum of 200 cells. Each cell which contained at least one particle is considered phagocytically active. Typically, 80-90 percent of the cells in control cultures ingested one or more particles.

Sample Preparation and Handling. All samples are tested in a concentration tested in duplicate. Solid samples are weighed directly into the culture vessels so that the final particle concentrations are 10, 30, 100, 300 and 1000 ug/ml of culture medium. Solid samples can be examined for the presence of leachable materials, if necessary. Liquid samples are added with and without sterile filtration to give a final concentration of 6, 20, 60, 200, and 600 ul/ml (10X medium is used in order to add as much sample as possible). The pH of the final incubation mixture is recorded before and after incubation. No pH adjustments are made for the initial testing. When pH adjustments are made, the sample is tested both with and without adjustment.

Results. Samples found in the initial screening to significantly affect the parameters being measured are retested for confirmation.

All of the above determinations are performed in duplicate. Since cell viability could be considered a binomial response, the arc-sine transformation is employed in the regression analysis. This technique helps to linearize the data when viability, as a percentage, is plotted versus the natural logarithm of the molar concentration. For data, the concentration of the test compound that yielded a 50 percent response for any test parameter (EC_{50}) should be obtained through inverse prediction of the simple regression line.

3.3.2.2 Human Lung Fibroblast (WI-38) Assay

Materials and Methods. Human lung fibroblasts can be obtained from the American Type Culture Collection, Rockville, Maryland, and should be maintained in 75 cm^2 Falcon flasks. Cultures are subcultivated twice weekly by use of 0.25 percent trysin in Gibco solution A with a 1:2 split ratio. Cultures should not be employed beyond the 35th subcultivation. Cultures for experimentation are seeded at 1.73×10^5 cells/ml (4.0 ml total volume) in 25 cm^2 Falcon flasks and maintained in Basal Medium Eagle (BME) with

Earles salts plus 10 percent fetal bovine serum (virus screened) 2 mmole/ml L-glutamine, 100 units/ml penicillin, 100 ug/ml streptomycin, and 2.5 ug/ml amphotericin-B. Cells maintained under these conditions show a period of rapid growth from 24 of 72 hours after subcultivation during which time the experiments are performed. Routinely antibiotics should be removed from the maintenance to determine the presence of contaminating microorganisms and mycoplasma.

The growth inhibition assay is performed by planting 1.5 to 2.0 x 10^5 cells per flask in 25 cm^2 Falcon flasks and adding dilutions of the effluent test material 24 hours after the cells have adhered to the flask surface. The cultures are incubated with closed caps for 20 hours at 37 C. At the end of this incubation period, the cells are trypsinized and and cell counts, cell viability, protein and ATP determinations are performed as described previously.

Sample Handling and Preparation. All samples are tested in a concentration response fashion (as described previously for the rabbit alveolar macrophages) with each concentration tested in duplicate.

Results. See description for the rabbit alveolar macrophage test.

3.3.2.3 Clonal Toxicity Assay

The clonal assay (e.g. using L-929, CHO or other suitable cell) involves the plating of specified numbers of cells (generally 100 to 1000 in increments of 100) per 100 mm or 60 mm tissue culture dish and the attachment of these cells for a 24-hour period. Replicate plates then are exposed to particulate or soluble (aqueous or limited organic) toxicants for 24-48 hours. The cultures then are washed free of toxicant, refed with fresh medium, and allowed to develop discrete "clonal" colonies of cells. After 10-16 days (time depends upon the cell line) the cultures are fixed and stained.

3.3.3 Acute In Vivo Test in Rodents (References 13-14)

Since the major objective of the Level 1 biological testing procedure is to identify toxicology problems at minimal cost, it is recommended that a two-step approach be taken to the initial acute *in vivo* toxicology evaluation of unknown compounds. The first is based on the quantal (all-

or-none) response and the second on the quantitative (graded) response. Normally, the quantal test is used to determine the necessity to carry out the quantitative assay.

3.3.3.1 Method Description

Quantal. Five male and five female young adult rats (approximately 250 g) will be purchased from the supplier and conditioned at the laboratory for a minumum of five days. A single dose of the test material undiluted if a liquid, diluted with a biologically inactive solvent if a solid will be administered by gavage to this population of animals in a single dose of 10 ml/kg (if a solvent is used for a solid, the diluent utilized should be the minimum quantity to effectively administer the test substance). Immediately following administration of the test substance and at frequent intervals during the first day, observations will be recorded on all toxic signs or pharmacological effects (Table 4). The frequency and severity of the signs will be scored. Particular attention will be paid to time of onset and disappearance of signs. Daily observations will be made on all animals through a 14 day observation period. Effluent samples which produce harmful effects in vivo and do not result in deaths, may be further investigated. At termination of the observation period, all surviving animals will be killed and necropsies will be performed. Similarly, necropsies will be performed on all animals that die during the course of this study.

Should no mortality occur in the quantal study, no further work need be done on the test substance and the LD50 should be reported as greater than 10 g/kg.

Quantative. If a single animal in the quantal study dies in the 14 day observation period, then a quantitative study will be performed. Eighty animals (See 3.3.3.1) equally divided by sex will be used for this study and maintained for 7 days in quarantine. Having determined good health in the study population, the animals will be randomly divided into four groups of five male and five female animals per group. The test substance treated as before will be administered in graded dosages according to the following schedule: 3.0, 1.0, 0.3, and 0.1 g/kg. A different dosage schedule may be selected depending on the results of the quantal study in relationship to the numbers of animals that died and severity and

TABLE 4. PHYSICAL EXAMINATIONS IN ACUTE TOXICITY TESTS IN RODENTS

Organ System	Observation and Examination	Common Signs of Toxicity
CNS and somatometer	Behavior	Change in attitude to observer, unusual vocalisation, restlessness, sedation
	Movements	Twitch, tremor, ataxia, catatonia, paralysis, convulsion, forced movements
	Reactivity to various stimuli	Irritability, passivity, anesthesia, hyperaesthesia
	Cerebral and spinal reflexes	Sluggishness, absence
	Muscle tone	Rigidity, flaccidity
Autonomic nervous system	Pupil size	Myosis, mydriasis
	Secretion	Salivation, lacrimation
Respiratory	Nostrils	Discharge
	Character and rate of breathing	Bradypnoea, dyspnoea, Cheyne-Stokes breathing, Kussmaul breathing
Cardiovascular	Palpation of cardiac region	Thrill, bradycardia, arrhythmia, stronger or weaker beat
Gastrointestinal	Stents	Diarrhea, constipation
	Abdominal shape	Flatulence, contraction
	Feces consistency and color	Unformed, black or clay colored
Genitourinary	Vulva, mammary glands	Swelling
	Penis	Prolapse
	Perineal region	Soiled
Skin and fur	Color, turgor, integrity	Reddening, flaccid skinfold, eruptions, piloerection
Mucous membranes	Conjunctiva, mouth	Discharge, congestion, hemorrhage cyanosis, jaundice
Eye	Eyeball	Exophthalmus, nystagmus
	Transparency	Opacities
Others	Rectal or paw skin temperature	Subnormal, increased
	Injection site	Swelling
	General condition	Abnormal posture, emaciation

type of signs. Observations and duration of study as well as necropsy procedure will be carried out as indicated above. The LD50 will be calculated by the method of Horn (Reference 14). If the data are not suitable for calculation of a precise LD50, i.e., total mortality occurs in the low dosage level, an estimate of the LD50 should be made or the LD50 could be expressed as greater than 3 g/kg or less than 0.1 g/kg. Occasionally it may be necessary to do higher dosage, lower dosages, or another series at intermediate dosages depending on the results of the above data at the discretion of the Project Officer.

3.3.3.2 Reports

Reports will be provided giving a statement of the methods used, the results obtained, and a statement of conclusions reached with regard to the toxicity of the test substances.

3.3.3.3 Discussion

Due to the complex mixture of chemical compounds in the sample and its subsequent potential additive or synergistic action, the rodent *in vivo* screen is one of the necessary test procedures.

Because of the availability of uniform strains of mice and rats, ease of housing, size, relatively low cost, and a large amount of published toxicologic data, these two species are usually the animals of choice for the measurement of acute toxicity.

The advantages of the *in vivo* toxicity assays are embodied mainly in the fact that the toxicological assessment is performed in whole animals. There is a significant background of test data on a wide range of toxicants for the rodent systems, thus supplying needed information for reliable interpretation of results with complex effluents.

The disadvantages of the acute rodent toxicity studies are that they by definition may not satisfactorily predict long-term/low-level exposures to toxic materials. An additional consideration is the need for multi-gram quantities of test material which may prohibit testing of small amounts of relatively purified toxic components of complex mixtures such as found in the particulate and gaseous samples.

3.4 ECOLOGICAL EFFECTS TESTS

3.4.1 Freshwater Algal Assay Procedure: Bottle Test (References 14, 15)

3.4.1.1 Introduction

An algal assay is based on the principle that growth is limited by the nutrient that is present in shortest supply with respect to the needs of the organism. The test is designed to be used to quantify the biological response (algal growth) to changes in concentrations of nutrients and to determine whether or not various effluents are toxic or inhibitory to algae. These measurements are made by adding a selected test alga to the test water and determining algal growth at appropriate intervals. Several methods that may be used for determining growth are listed in the results section.

3.4.1.2 Method Description

Apparatus

Constant temperature room or equivalent incubator arrangement capable of providing temperature control at 24 ± 2 C.

Illumination - "cool white" fluorescent lighting to provide 4,304 lux (400 ft-c) ± 10 percent or 2,152 lux (200 ft-c) ± 10 percent measured adjacent to the flask at the liquid level.

Shaking apparatus for test culture flasks capable of 110 oscillations per minute.

Analytical balance capable of weighting 100 g. with a precision of ± 0.1 mg.

Oven - dry heat capable of temperature of 220 ± 1 C.

Culture flasks - Erlenmeyer, Pyrex or Kimax type glass. While flask size is not critical due to CO_2 limitation, the surface to volume ratios are. Recommended ratios are 40 ml of sample in 125 ml flask.

Culture flask closures - foam plugs, loose-fitting aluminum foil, or inverted beakers.

Millipore filter apparatus - for use with 47-mm pre-filter pads and 0.45μ porosity membrane filters.

Autoclave or pressure cooker - capable of producing 1.1 kg/cm^2

(15 psi) at 121C.

Sampler - non metallic.

Sample bottles - borosilicate glass, linear polyethylene, polycarbonate or polypropylene, capable of being autoclaved.

Light meter capable of being calibrated against a standard source or light meter.

Microscope and illuminator - good quality, general purpose.

Haemocytometer or plankton counting slide.

Centrifuge capable of a relative centrifugal force of at least 1,000 G.

pH meter having a scale of 0 - 14 pH units with an accuracy of ± 0.1 pH unit.

Spectrophotometer or colorimeter for use at 600 to 750 mμ.

Pipettes - pre-sterilized, disposable.

Reagents

Algal Nutrient Medium - Prepare a stock solution of each of the macronutrient salts listed in Table 5 in 1000 times the specified final concentrations in glass distilled and/or deionized water.

TABLE 5. MACRONUTRIENTS NEEDED FOR ALGAL NUTRIENT MEDIUM

Macronutrient Compound	Stock Solution Concentration mg/l	Elements	Resulting Concentrations mg/l
$NaNO_3$	25,500	N	4,200
		Na	11,001
K_2HPO_4	1,044	P	0,186
$MgCl_2$	5,700	Mg	2,904
$MgSO_4 \cdot 7H_2O$	14,700	S	1,911
$CaCl_2 \cdot 2H_2O$	4,410	Ca	1,203
$NaHCO_3$	15,000	K	0,488

Prepare a single stock mix of the micronutrients listed in Table 6 at 1000 times the final specified concentrations in glass distilled and/or deionized water. The trace metal $FeCl_3$ and the EDTA must be combined in a single stock mix at 1000 times the final concentrations.

TABLE 6. MICRONUTRIENT STOCK SOLUTION

Compound	Concentration mg/l	Element	Resulting Concentration mg/l
H_3BO_3	185.64	B	33.0
$MnCl_2$	266.37	Mn	116.0
$ZnCl_2$	35.70	Zn	15.0
$CoCl_2$	0.78	Co	.35
$CuCl_2$	0.009	Cu	.003
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	3.88
$FeCl_3$	96.0	Fe	33.0
$Na_2EDTA \cdot 2H_2O$	1000.0	Fe	33.0

Preparation of Glassware. Carefully check all glassware for cracks or leakage around screw-caps and thoroughly wash it in hot lab soap or phosphorus-free detergent. The laboratory sink should be cleaned previously with hot soap and water and rinsed with 20 percent hydrochloric acid to remove residual chemicals or other contaminants. Following washing, rinse glassware well in hot tap water and give at least one full rinse in 20 percent concentrated HCl solution. Rinse all glassware thoroughly at least five times in distilled and deionised water.

Sterility Techniques. When bacterial growth may interfere with the test, use the following sterile techniques for preparing the culture medium.

1. Sterilise a suitable quantity of double-distilled water.
2. Prepare suitable autoclave-sterilised containers.
3. Pre-rinse sterile 0.22 μ porosity membrane filters by passing sterile double-distilled water through them.

Preparation of Algal Culture Medium. Prepare medium by adding 1 ml of each of the macronutrient stock solutions in the order listed to 900 ml of glass distilled and/or deionised water, mixing between each

addition. Filter-sterilize by passing through the sterile, pre-rinsed, 0.22μ porosity membrane filter into the sterile container. Add 1 ml of the micronutrient stock solution and 1 ml of the $FeCl_3$-EDTA mixture which have been sterilized in the same manner and then make up to 1 liter with sterile distilled and/or deionized water.

When additional sterile procedures are required, consult Reference 13.

Recommended Test Algae

Selenastrum capricornutum Printz., 10^3 cells/ml

Microcystis aeruginosa Kutz. emend Elenkin (Anacystis cyanea Drouet and Daily, 50 x 10^3 cells/ml

Anabaena flos-aquae (Lyngb.) Debrebisson, 50 x 10^3 cells/ml

Diatom - Cyclotella sp. 10^3 cells/ml

Nitzschia sp. 10^3 cells/ml

These algae are recommended because they provide a representative cross-section of the types of algae likely to be found in waters of differing nutritional status. Other algae that may be used for special test purposes include diatoms where they may be a consideration in relation to water supply problems and naturally occurring mixtures of species.

Algal cultures may be secured from National Eutrophication Research Program, Pacific Northwest Environmental Research Laboratory, U.S. Environmental Protection Agency, 200 S.W. 35th Street, Corvallis, Oregon 97330, and from Department of Botany, University of Indiana, Bloomington, Indiana.

Maintenance of Stock Cultures. Culture S. capricornutum and diatoms under continuous, "cool white" fluorescent lighting at 4304 lux (400 ft-c) ± 10 percent at 24 ± 2 C, and M. aeruginosa and A. flos-aquae at 2152 lux (200 ft-c) ± 10 percent; shake at 110 oscillations per minute.

After receiving, transfer the inocula species aseptically to filter-sterilized algal nutrient media (e.g., 1 ml of inoculum to 30 ml of algal nutrient medium in 125 ml Erlenmeyer flask or similar ratios of inoculum to medium in larger flasks). Repeat daily thereafter for 7 days. On the eighth day, the initial transfer culture will be 7-days old and this culture will then be used as the inoculum for the next transfer. Thereafter, only 7-day old cultures will be used for inocula. These stock cultures should be incubated under the same conditions as prescribed for the test flasks.

Procedures

Sampling and Storage. Samples for the test may be (1) surface samples from lakes, rivers, or other bodies of water; (2) wastewaters; (3) substances of concern that may ultimately reach surface waters, and (4) any sample to which nutrients or other substances are added or from which they are removed.

In the case of surface waters, collect them directly in polyethylene containers leaving a minimum of air space. Samples from desired depth should be collected with a non-metallic water sampler bottle and then transferred to polyethylene bottles. Samples of other substances to be tested should be collected and stored in containers fabricated of materials that do not alter the character of the sample.

Transport samples to the laboratory in the dark and at ice temperature. Temporary storage in the laboratory should occur under similar conditions.

Preparation of Filtered Sample. As soon as possible, but not to exceed 2 days after collection, begin the test procedure. Test each sample in triplicate, but for statistical purposes divide each into three aliquots before filtration and thereafter treat as separate samples. Pretreat the 0.45μ porosity membrane filter by passing 50 ml of distilled water through it and discard the filtrate. Then, filter a quantity of the sample as needed under reduced pressure of 0.5 atmosphere. Use the resulting filtered sample water in testing. If suspended matter in the sample requires it, the 0.45μ porosity membrane filter should be preceded by an appropriate pre-filter pad (for example, fiber glass) which is also pretreated as described above.

Preparation of Inoculum. After no less than three and no more than seven weekly transfers, use the cultures to inoculate the test waters. Centrifuge cells from a 7-day old stock culture and discard the supernatant. Resuspend the sedimented cells in an appropriate volume of a 15,000 mg/l $NaHCO_3$ solution and recentrifuge. Then resuspend sedimented cells in the $NaHCO_3$ solution and count. Pipet a portion into each test water as the inoculum.

Amount of Inoculum. Count the cells suspended in the bicarbonate solution and pipet into the test water to give a starting cell concentration in the test waters as follows:

S. capricornutum 10^3 cells/ml

M. aeruginosa 50×10^3 cells/ml

A. flos-aquae 50×10^3 cells/ml

Diatoms 10^3 cells/ml.

The volume of the transfer is calculated to result in a concentration of 10^3 cells/ml in the test flasks (e.g., 3×10^5 cells/ml in stock culture requires a 0.3 ml transfer per 100 ml test water). Transfer should not exceed 1 ml per 100 ml (a minimum 1×10^5 cells/ml in stock culture is required).

Preparation of Test Flasks. Add filtered sample water in equal amounts to the sets of test flasks. The amount of sample water added is dependent upon the size of the test flask used, but should be such that good agitation is realized when shaking (e.g., a 30 ml water sample in a 125 ml Erlenmeyer flask).

Simultaneously, test a triplicate set of flasks containing the algal nutrient medium for algal growth comparison. These flasks should contain the same volume of medium and inoculum as the flasks containing the sample water.

Depending upon the type and amount of information being sought, incorporate other sets of test flasks for comparison. These may include such as the following:

- Sample water supplemented with phosphorus (Example: 0.1 mg P/l),
- Sample water supplemented with nitrogen (Example: 1.0 mg N/l),
- Sample water supplemented with phosphorus and nitrogen (Example: 0.1 mg P/l and 1.0 mg N/l),
- Sample water diluted with distilled water to give various concentrations (Example: 10 and 50 percent dilutions).

Incubation. Incubate test flask cultures at the following controlled environmental conditions:

- Temperature - 24 C ± 2 C
- Illumination - continuous "cool white" fluorescent lighting to provide 100 ft-c (1076 lux) at midpoint (from top to bottom) of culture flasks of Anabaena and and Microcystis and 400 ft-c (4304 lux) of culture flasks of Selenastrum.

- Shaking - continuous reciprocating or gyratory, 60 strokes per minute.

3.4.1.3 Results

Observations. Depending upon the method for evaluating growth, choose the method used to determine growth response during incubation from among the following. Count cells on a microscope, using a hemacytometer or a Palmer-Maloney or Sedgwick-Rafter plankton counting chamber. Determine the amount of algal biomass by measuring the absorbance of the culture at 600 to 750 mμ with a colorimeter or spectrophotometer. Measure the amount of chlorophyll contained in the algae either directly (in vivo) by fluorometry or after extraction. An electronic particle counter provides an accurate and rapid count of cells. All methods used for determining the algal biomass should be related to a dry weight measurement (mg/l) determined gravimetrically.

Growth Measurement. Two parameters are used to describe the growth of each test alga: maximum specific growth rate and maximum standing crop. The maximum specific growth rate (μ max) for an individual flask is the largest specific growth rate (μ) occurring at any time during incubation. The μ max for a set of replicates is determined by averaging the μ max of the individual flasks. The specific growth rate, μ, is defined by

$$\mu = \frac{\ln (X_2/X_1)}{t_2 - t_1}$$

where,

X_2 = biomass conc. at the end of the selected time interval

X_1 = biomass conc. at the beginning of the selected time

$t_2 - t_1$ = elapsed time (days) between selected determination of biomass.

Because the maximum specific growth rate (μ max) occurs during the logarithmic phase of growth (usually between day 0 and day 3), the biomass must be measured at least daily during the first 3 days of incubation.

The maximum standing crop in any flask is defined as the maximum algal biomass (dry weight) achieved during incubation. For practical purposes, the maximum standing crop can be assumed to have been achieved when the rate of increase in biomass has declined to less than 3 percent per day.

After the maximum standing crop has been achieved, determine the dry weight of the algal biomass gravimetrically by either the aluminum dish or filtration technique. If biomass is determined indirectly, convert the results to an equivalent dry weight using appropriate conversion factors.

Following determination of the maximum standing crop and maximum specific growth rate, compare these values for the effluent tests and standard culture medium tests (controls) to determine the EC50. The EC50 represents that concentration at which either 50 percent of the maximum standing crop or maximum specific growth rate is obtained, as compared with controls.

Calculation of the EC50. The EC50 is the concentration at which growth is 50 percent of the control. It may be estimated by interpolation by plotting the data on semilogarithmic coordinate paper with concentrations on the logarithmic axis and percentage growth on the arithmetic axis. A straight line is drawn between the two points on either side of the 50 percent growth value. The concentration at which the line crosses the 50 percent growth line is the EC50 value.

Stimulation. If growth was stimulated by the effluent, report the percentage stimulation at each concentration.

Cell Count Measurement. Any method of count (i.e., cell number/ml) determination is applicable, including direct microscopic counting (hemocytometer), Palmer-Maloney slide, or the use of electronic particle counters. Make counts at least every other day during log-phase growth. These may be less frequent after the log phase. Preferred counting days are: 3, 5, 7, 9, 13, 17, and 21. For detailed procedures of plankton counting and chlorophyll analysis, see Reference 15.

Gravimetric Procedure. This method is particularly useful for assessing the growth of Anabaena flos-aquae. The cells of the alga grow in filaments and it is difficult to obtain accurate cell counts. The method may also be used with S. capricornutum, M. aeruginosa, and other species of algae. In any case, use it only with either relatively dense cultures or large volumes of thinner cultures. Otherwise, the error may be large. Two methods may be employed.

Method 1. Centrifuge a suitable portion of algal suspension. Use a continuous-flow centrifuge, if available. Discard the supernatant, resuspend the sedimented cells in distilled water, transfer to tared

crucibles or aluminum cups, dry overnight in a hot air oven at 105 C, cool in a desiccator over Drierite for 1 hour, and weigh.

Aluminum cups should be pretreated before taring by adding approximately 15 ml of distilled water and drying at 105 C in the oven. Repeat this pretreatment three times; then the cups can be tared accurately.

Method II. This method involves filtering a measured portion of algal suspension through a tared Millipore® filter, preferably a type AA filter with an 0.80 micron pore size.

The method is as follows:

a. Dry filters for several hours at 90 C in an oven.(1) The filters may be placed in folded sheets of paper upon which the weights or codes may be written.

b. Cool filters in a desiccator containing desiccant.

c. Filter a suitable measured aliquot of the culture under a vacuum of 0.5 atmosphere. Normally 10 ml is sufficient, but in thin cultures more may be required.

d. Rinse the filter funnel with 30 ml distilled water using a wash bottle and allow the rinsing to pass through the filter. This serves to transfer all of the algae to the filter and to wash the nutrient salts from the filter.

e. Dry the filter in its paper folder at 90 C, cool in the desiccator, and weigh.(2)

f. To collect for loss of weight of filters during washing, wash two blank filters with 30 ml of distilled water, pouring it through slowly under reduced vacuum. Dry and weigh filters and record weight loss. This correction is now large, but is essential for meaningful results on thin cultures. For example, if 10 ml have been filtered and yield a difference between tare and final of 1.10 mg and the blank has lost 0.02 mg, then the culture contains (1.10 + 0.02) x 100 = 112 mg/l dry weight.

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1. The drying temperature has been selected to avoid damage to filters.

2. In weighing, do not hang the filters over the edge of the pan or electrostatic attraction of the filter to the balance will result in an error.

Absorbance Measurements. Measure absorbance initially and after each 24 hours with a spectrophotometer or colorimeter at a wavelength of 600 mμ. In reporting results, specify the instrument make or model, the geometry and path length of the cuvette, and the equivalence between absorbance and some other measure of cell quantity.

Absorbance, as defined by the Beer-Lambert Law, $A = \log\frac{I_0}{I_s} = abc$(1) ($I_0$ = intensity of incident light, I_s = intensity of transmitted light, a = absorptivity index characteristic for the solution, b = length of cell path, c = concentration of solute in solution) is usually derived for absorption of light by molecules of solute in homogeneous solution. It can be derived also for a suspension of uniform particles, but with some necessary added restrictions. For particles of bacterial or algal cell size, I_s is less than I_0 by virtue of absorption and also by virtue of scattering caused by cell reflections and refractions. The fraction of the latter which reaches the light measuring receiver depends upon the instrument design. A large receiver close to the cuvette catches much of the scattered light (i.e., is insensitive to scattering). A small receiver far from the cuvette in a long-focused or diaphragmed optical path catches very little scattered light (i.e., is very sensitive to scattering) (Ref. 14).

Most current instruments are likely to be more sensitive to scattering than to absorption as evidenced by effect of wavelength. A simple test is: for a green alga, light absorption by its pigments *in vivo* will show relative absorbance ratios such as 70: 500: 1, for 600: 680: 750 mμ light (ratios probably correct in order of magnitude). In practical measurements, without elaborate precautions to avoid effects of scattering, the ratios will always be very much less.

In any photometric measure of absorbance, considerations of precision lead to a simple rule of thumb that measurements be limited to a range of $0.05 < A < 1.0$. A further reason for this restriction is that for satisfactory suspension, the linearity between A and concentration holds practically only over an limited range. Hence, values measured should be limited to this range by concentration or dilution before measurement. If equipment permits, adjustments in cell path are better than concentration.

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(1) In former terminology absorbance (A) = optical density (D) = Gel.

A common and necessary check upon instrumentation is to measure A on various dilutions of an algal suspension. By this means absorbance can be calibrated against any other measure of cell quantity (X) such as cell number or dry weight. There is no assurance that the relation between A and X will be constant and independent of culture conditions. As noted above, the absorbance measured is a complex function of volume, size, and pigmentation of the cells. Hence, the relation between A and X should be examined on different batches of algae which best simulate actual conditions of the test.

Report. In the final report, include the following specific information as well as the general information provided in all analyses (see Section 3.5):

1. The EC50 at 12 days and other days of importance to be decided upon by the shape of the growth curve.
2. The specific growth rate between days 3 and 12 and any other period that should be reported depending upon the shape of the growth curve.

3.4.1.4 Discussion

The bottle test is the only algal bioassay that has undergone sufficient evaluation and refinement to be considered reliable. Following years of intensive evaluation, participating laboratories, have obtained excellent agreement in results. For more detailed information concerning bioassays, consult Reference 15.

3.4.2 Acute Static Bioassays With Freshwater Fish and Daphnia (References 16-34)

3.4.2.1 Introduction and Rationale

Biological testing must be considered as well as chemical and physical parameters when assessing the potential impact of complex industrial or a combination of municipal and industrial wastes on the aquatic environment. Biological testing usually involves performing toxicity tests on samples of treated wastes.

In a toxicity test, two or more treatments are used to study the effect of a toxic agent on test organisms which are usually all of the same species. Aquatic organisms will integrate synergistic and antagonistic effects of all the components over the duration of the exposure.

Although toxicity tests with aquatic organisms can be conducted by applying the toxic agent directly to the test organisms, such as by injection or in food, most tests are conducted by exposing the test organisms to test solutions containing various levels of a toxic agent. One or more control treatments are used to provide a measure of the acceptability of the test by giving some indication of the healthiness of the test organisms and the suitability of the dilution water, test conditions, handling procedures, etc. A control treatment is an exposure of the test organisms to dilution water with no toxic agent added. The other treatments are exposures of the test organisms to dilution water with toxic agent added. Generally the most important data obtained from a toxicity test are the percentages of test organisms that are affected in a specified way by each of the treatments. The result derived from these data is a measure of the toxicity of the toxic agent to the test organisms under the conditions of the test or, in other words, a measure of the susceptibility of the test organisms to the toxic agent.

Acute toxicity tests are generally used to determine the level of toxic agent that produces an adverse effect on a specified percentage of the test organisms in a short period of time. Because death is normally an easily detected and obviously important adverse effect, the most common acute toxicity test is the acute mortality test. Experimentally, 50 percent effect is the most reproducible measure of the toxicity of a toxic agent to a group of test organisms, and 96 hours is often a convenient, reasonably useful exposure duration. Therefore, the measure of acute toxicity most often used with fish and macroinvertebrates is the 96-hour median lethal concentration (96-hr LC50). Thus the result of an acute mortality test is the statistically derived best estimate of the LC50, which is the concentration of toxicant in dilution water that is lethal to exactly 50 percent of the test organisms during continuous exposure for a specified period of time, based on data from one experiment. However, the measure of acute toxicity most often used with daphnids and midge larvae is the 48-hour median effective concentration (48-hr EC50) based on immobilization. The terms median lethal concentration (LC50) and median effective concentration (EC50) are consistent with the widely used terms median lethal dose (LD50) and median effective dose (ED50), respectively. However, whereas "concentration" refers to the concentration of toxicant in the test solution, "dose" refers to the amount of toxicant that enters the test organism.

The static technique provides the easiest measure of toxicity and is recommended for the waste assessment ... fies. Fathead minnow (Pimephales promelas) and Daphnia pulex are the test animals.

The following procedure is based primarily on the methods given in Reference 19. In many instances, sentences and/or complete paragraphs are used verbatim.

The primary objections to the following procedure are that the recommended dilution water may not closely simulate receiving water characteristics and the fathead minnow may not be representative of the most sensitive species in a given geographical area. However, the procedure will adequately serve to develop relative toxicity data for the purpose of ranking industries based on the toxicity of their effluents.

3.6.3.2 Method Description

Equipment

Facilities. The facilities should include tanks for holding and acclimating test organisms, and a constant-temperature area or recirculating water bath for the test chambers. There should be a dilution water tank that may be used to prepare reconstituted water. This is sometimes elevated so dilution water can flow by gravity into holding and acclimation tanks and test chambers. Holding, acclimation, and dilution water tanks should be equipped for temperature control and aeration. Air used for aeration must be free of oil and fumes; filters to remove oil and water are desirable. During holding, acclimation, and testing, test organisms should be shielded from disturbances. The test facility must be well ventilated and free of fumes. A 16-hour light and 8-hour dark photoperiod should be provided.

Construction Materials. Construction materials and commercially purchased equipment that may contact any water into which test organisms are placed should not contain any substances that can be leached or dissolved by the water. In addition, materials and equipment that contact stock solutions or test solutions should be chosen to minimize sorption of toxicants from water. Glass, #316 stainless steel, and perfluorocarbon plastics must be used whenever possible to minimize leaching, dissolution, and sorption. Unplasticized plastics can be used for holding and acclimation tanks and in the water supply system. Rubber, copper, brass, and lead must not come in contact with dilution water, stock solutions, effluent samples, or test solutions.

Test Chambers. Test chambers for the fathead minnow should be 19.6-liter (five gallon), widemouth, soft-glass pickle jars containing 15 liters of solution, or 30 cm x 60 cm x 30 cm deep, all-glass chambers. The all-glass chambers can be made by welding, not soldering, stainless steel or by gluing double-strength or stronger window glass with clear silicon adhesive. Silicon adhesive sorbs some organochlorine and organophosphorus pesticides which are then difficult to remove. Therefore, as little of the adhesive as possible should be in contact with water; extra beads of adhesive should be on the outside of chambers rather than on the inside. The test solution should be between 15 and 20 cm deep.

Daphnids may be exposed in 3.9-liter (1-gallon), widemouth, soft-glass bottles, or battery jars containing 2 to 3 liters of solution. Loosely covered 250-ml beakers containing 200 ml of solution may also be used.

Cleaning. Test chambers must be cleaned before use. New ones must be washed with detergent and rinsed with 100 percent acetone, water, acid (such as 3 percent concentrated nitric acid), and twice with tap or other clean water. At the end of every test, if the test chambers are to be used again, they should be (a) emptied, (b) rinsed with water, (c) cleaned by a procedure appropriate for removing the constituent tested (e.g., acid to remove metals and bases; detergent, organic solvent, or activated carbon to remove organic compounds), and (d) rinsed twice with water. Acid is useful for removing mineral deposits, and 200 mg of hypochlorite/liter is useful for removing organic matter and for disinfection. However, acid and hypochlorite must not be used together. Test chambers must be rinsed with dilution water just before use.

Dilution Water

General Requirements. An adequate supply of a dilution water that is acceptable to the test organisms and the purpose of the test must be available. For acute toxicity tests, a minimal criterion for an acceptable dilution water is that healthy test organisms will survive in it for the duration of acclimation and testing without showing signs of stress, such as discoloration or unusual behavior. Because daphnids are more sensitive to many toxicants than most other freshwater aquatic animals, a more realistic criterion for an acceptable freshwater dilution water is that first instar daphnids will survive in it for 48 hours without food. A more stringent criterion for an acceptable dilution water is that test organisms will survive, grow, and reproduce satisfactorily in it. Water in which daphnids

will survive and reproduce satisfactorily should be an acceptable dilution water for most tests with freshwater animals. The dilution water should be intensively aerated prior to the introduction of the toxicant. Adequate aeration will bring the pH and the concentration of dissolved oxygen and other gases into equilibrium with air, and will minimise oxygen demand and the concentration of volatiles. The concentration of dissolved oxygen in the dilution water should be between 90 percent and 100 percent saturation, and water that may be contaminated with undesirable microorganisms should be passed through a properly maintained ultraviolet sterilizer equipped with an intensity meter.

Recommended Dilution Water. The dilution water should be of constant quality and should contain less than the following maximum accepted levels of substances:

Suspended solids	20 mg/l
Total organic carbon	10 mg/l
Un-ionized ammonia	20 ug/l
Residual chlorine	1 ug/l
Total organophosphorus pesticides	50 ng/l
Total organochlorine pesticides plus PCB's	50 ng/l

The dilution water is considered to be constant quality if the monthly ranges of the hardness, alkalinity, and specific conductance are less than 10 percent of their respective averages and if the monthly range of pH is less than 0.4 units. The dilution water should be obtained from an uncontaminated well or spring if possible; only as a last resort should dechlorinated water be used. If dechlorinated water is used, it either must be shown that first instar daphnids can survive in it for 48 hours without food or residual chlorine must be measured. When possible, dilution water with a hardness of 100 mg/l $\pm$ 10 percent as $CaCO_3$ should be used to minimise problems that occur in data interpretation and comparison.

Test Organisms

Species. The fathead minnow Pimephales promelas should be the primary animal used in all tests carried out under this protocol for the environmental assessment studies. A secondary choice is Daphnia pulex, which should be used if additional toxicity data are desired or if it is impossible to use the fathead minnow. These species were chosen for their ready availability, heartiness, and the ease, convenience, and economy with which they can be maintained in the laboratory.

Source. Local sources of fish are private, state, and federal fish hatcheries. Fathead minnows may be captured from wild populations in relatively unpolluted areas. However, collecting permits may be required by local and state agencies. Fish collected by electroshocking should not be used. Daphnia should be reared in the testing facility from laboratory cultures. Daphnia from cultures in which ephippia are being produced should not be used. All animals in a test should be from the same source and as healthy and uniform in size and age as possible.

Size. Very young (not yet actively feeding), spawning, or spent fish should not be used in the testing being done using this protocol. The fish should weigh between 0.5 and 1.0 g. In any single test, all fish should be from the same year class, and the standard length (tip of snout to end of caudal peduncle) of the longest fish should be no more than twice that of the shortest fish.

The Daphnia pulex should be in the first instar stage of their life cycle.

Care and Handling. To avoid unnecessary stress, organisms should not be subjected to rapid changes in temperature or water quality. In general, aquatic organisms should not be subjected to more than a 3 C change in water temperature in any 12-hour period. Holding and acclimation tanks should be sterilized with an iodophor or with 200 mg of hypochlorite/liter for 1 hour, scrubbed well once during the hour, and then rinsed well between groups of test organisms. When organisms are first brought into the facility, they should be quarantined at least until they appear to be disease free. To maintain organisms in good condition during holding and acclimation, crowding should be avoided and the dissolved oxygen concentration must be maintained between 60 and 100 percent saturation; gentle aeration may be used if necessary. Organisms should be fed at least once a day and the tanks scrubbed at least twice a week. Careful observations should be made during holding and acclimation for signs of disease, stress, physical damage, and mortality. Dead and abnormal individuals must be discarded.

Organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible, so that the organisms are not unnecessarily stressed. Organisms that touch dry surfaces or are dropped or injured during handling must be discarded. Small dip nets are best for handling the fish. These are commercially avail-

able, or they can be made from small-mesh nylon netting, nylon or silk bolting cloth, plankton netting, or similar material. Smooth glass tubes with rubber bulbs should be used for transferring the Daphnia. Equipment used to handle aquatic organisms should be sterilized between uses with an iodophor, 200 mg of hypochlorite/liter, or 30 percent formalin plus 1 percent benzalkonium chloride. Hands should be washed or sterilized before handling or feeding test organisms.

Disease Treatment. Freshwater fish may be chemically treated to cure or prevent diseases by using the treatments recommended in Table 7; but if they are severely diseased, it is often better to destroy the entire lot. Until acceptable treatments have been proven effective, all other diseased animals should be discarded. Generally the fish should not be treated during the first 16 hours after arrival at the facility because they are probably stressed due to collection or transportation and some are treated during transit. Tests must not be begun with treated fish for at least 4 days after treatment. Tanks and test chambers which may be contaminated with undesirable microorganisms should be sterilized for 1 hour with an iodophor or with 200 mg of hypochlorite/liter.

Holding and Acclimation. After collection or transportation, the fish or Daphnia should be held in and acclimated to the dilution water for at least 2 days before beginning a test. They should be held if possible under stable conditions of temperature and water quality in a flow-through system with a flow rate of at least two water volumes per day or in a recirculating system in which the water flows through a carbon filter and an ultraviolet sterilizer. When possible, the fish should be held in the dilution water at the temperature at which they are to be tested. During long holding periods, however, it is generally easier and safer to hold them at a lower temperature (10-15 C) rather than higher temperatures (20-25 C) because the metabolic rate and the number and severity of disease outbreaks are reduced.

A group of animals must not be used for a test if individuals appear to be diseased or otherwise stressed or if more than 5 percent die during the 48 hours immediately prior to the beginning of the test. If a group fails to meet these criteria, all individuals must be discarded or treated and held an additional 4 days. Usually it is more practical to discard the entire group and begin over.

The fish should not be fed for 48 hours before the beginning of a test. However, the Daphnia may be fed up to the beginning of the test.

TABLE 7. RECOMMENDED PROPHYLACTIC AND THERAPEUTIC TREATMENTS FOR FRESHWATER FISH[a]

Disease	Chemical	Concentration (mg/l)	Application
External bacteria	Benzalkonium chloride (Hyamine 1622[c])	1-2 AI[b]	30-60 min[a]
	Nitrofurazone (water mix)	3-5 AI	30-60 min[a]
	Neomycin sulfate	33	30-60 min[a]
	Oxytetracycline hydrochloride (water soluble)	33 AI	30-60 min[a]
Monogenetic trematodes, fungi, and external protozoa[d]	Formalin plus zinc-free malachite green oxalate	33 0.1	1-2 hours[a]
	Formalin	150-250	30-60 min[a]
	Potassium permanganate	2-6	30-60 min[a]
	Sodium chloride	15,000-30,000 2000-4000	5-10 min dip e,a
	Dexol[e] (33% AI)	30	30-60 min[a]
Parasitic copepods	Trichlorfon (Masoten[f])	0.25 AI	f

[a]These recommendations do not imply that these treatments have been cleared or registered for these uses. Appropriate state and federal regulatory agencies should be consulted to determine if the treatment in question can be used and under what conditions the uses are permitted. These treatments should be used only on fish intended for research. They have been found dependable, but efficacy against diseases and toxicity to fish may be altered by temperature or water quality. Researchers are cautioned to test treatments on small lots of fish before making large-scale applications. Prevention of disease is preferred, and newly acquired fish should be treated with the formalin-malachite green combination on three alternate days if possible. However, generally fish should not be treated on the first day they are in the facility. This table is merely an attempt to indicate the order of preference of treatments that have been reported to be effective. Before a treatment is used, additional information should be obtained from sources such as Davis (1953), Hoffman and Meyer (1974), Reichenbach-Klinke and Elkan (1965), Sniesko (1970), and van Duijn (1973). (Ref. 20, 23, 25, 26, 32.)

TABLE 7. (Continued)

[b]AI = active ingredient.

[c]Treatment may be accomplished by (1) transferring the fish to a static treatment tank and back to a holding tank; (2) temporarily stopping the flow in a flow-through system, treating the fish in a static manner, and then resuming the flow to flush out the chemical; or (3) continuously adding a stock solution of the chemical to a flow-through system by means of a metered flow or the technique of Brungs and Mount (Ref. 16).

[d]One treatment is usually sufficient except for "Ich", which must be treated daily or every other day until no sign of the protozoan remains. This may take 4 to 5 weeks at 5 to 10 C and 11 to 13 days at 19 to 21 C. A temperature of 32 C is lethal to Ich in one week.

[e]Minimum of 24 hours but may be continued indefinitely.

[f]Continuous treatment should be employed in static or flow-through systems until no copepods remain, except that treatment should not be continued for over 4 weeks and should not be used above 27 C.

Test Procedure

Experimental Design. At least 10, but preferably 20, organisms must be exposed to each treatment. They may be divided between two or more test chambers. The use of more organisms and replicate test chambers for each treatment is desirable. Randomization of the treatments is desirable; if replicates are used, random assignment of one test chamber for each treatment in a row, followed by random assignment of a second test chamber for each treatment in another or an extension of the same row, is recommended rather than total randomization. A representative sample of the test organisms should be impartially distributed to the test chambers, either by adding one (if there are to be less than 11 organisms per container) or two (if there are to be more than 11 organisms per container) test organisms to each chamber, and then adding one or two more, and repeating the process until each test chamber has the desired number of test organisms in it. Alternatively, the organisms can be assigned either by random assignment of one organism to each test chamber, random assignment of a second organism to each test chamber, etc., or by total randomization. It is often convenient to assign organisms to other containers and then add them to the test chambers all at once.

Every test requires a control which consists of the same dilution water, conditions, procedures, and organisms as are used in the remainder of the test. If any additive is present in any of the test chambers, an additive control is also required. This additive control is treated the same as the regular control except that the highest amount of additive present in any other test chamber is added to this test chamber. A test is not acceptable if more than 10 percent of the organisms die in any control in a test determining an LC50 or show the effect in a test determining an EC50.

It is desirable to repeat the test at a later time to obtain information on the reproducibility of the results of the test.

Dissolved Oxygen Concentration. Aeration of the test solutions during the test should be avoided if possible. If necessary, aeration should be done with minimal agitation of the test solution.

Test Temperatures. The test with fathead minnows should be run at 22 C and with Daphnia at 17 C with no more deviation than 1 C during the test.

Loading. The grams of organism per liter of solution in the test chambers must not be so high that it affects the results of the test. Therefore the loading must be limited to insure that the concentration of dissolved

oxygen and toxicant is not decreased below acceptable levels, that the concentration of metabolic products does not increase above acceptable levels, and that the organisms are not stressed due to crowding. The loading in the test chambers must not exceed 0.3 g/liter. A lower loading rate should be used if low dissolved oxygen problems may occur. If the dissolved oxygen concentration is less than 60 percent saturation in any test chamber, it may be desirable to conduct the toxicity test with a modified procedure by slowly bubbling air or oxygen through the solutions in the test chambers during the test. If the modified procedure is used, the exact methodology must be described in detail in all reports of the test. In some instances, it may be necessary to run an aerated and a non-aerated test side by side.

Toxicant. The toxicant is a sample of an effluent. The sample of the effluent must not be aerated or altered in any way except that it may be filtered through a sieve or screen with 2 mm or larger holes. Samples must be covered at all times and violent agitation must be avoided. Undissolved materials must be uniformly dispersed by gentle agitation immediately before any aliquot of the sample is taken for use. The timing of the test and the collection of samples should be based on an understanding of the short and long-term operations and schedules of the discharger if possible.

Separate tests generally should be conducted on at least two grab samples, and more tests may often be desirable especially if there are known sources of variability such as process changes. Tests on composite samples may be desirable in some cases. Tests should be begun as soon as possible, but tests must be begun within 8 hours after the sample is obtained. The temperature of the sample should be adjusted to the test temperature (± 2 C) and maintained at that temperature until portions are added to the dilution water. Often it is convenient to store the sample in the constant temperature water bath or area in which the test chambers are placed during the test.

Beginning the Test. The animals should be put in the test chambers within 30 minutes after the effluent sample is added to the dilution water.

Feeding. The animals must not be fed while in the test chambers.

Duration. A test begins when the organisms are first exposed to the effluent. Daphnia must be exposed for 48 hours and fathead minnows for 96 hours. If time and resources permit, observations may be extended for longer time periods.

3.4.2.3 Results

Biological Data. The number of dead or affected organisms in each chamber must be counted every 24 hours after the beginning of the test. More frequent observations are desirable, especially near the beginning of the test. Dead animals must be removed as soon as they are observed.

Death is the adverse effect most often used to study acute toxicity with aquatic organisms. The criteria for death are usually the lack of movement, especially the absence of gill movement in fish, and the lack of reaction to gentle prodding. However, because death is not easily determined for some invertebrates, an EC50 is often determined rather than an LC50. The effect generally used to determine the EC50 for Daphnia is immobilization which is defined as the lack of movement except for minor activity of appendages. General observation of such things as erratic swimming, loss of reflex, discoloration, behavioral changes, excessive mucous production, hyperventilation, opaque eyes, curved spine, and hemorrhaging should be reported.

The weights and standard lengths of the fathead minnows should be determined by measuring representative animals before the test or the control animals after the test. Fish that are to be used in a test must not be weighed or measured after acclimation has begun.

Chemical and Physical Data. The dissolved oxygen concentration must be measured at the beginning of the test and every 24 hours thereafter to the end of the test in the control and the high, medium, and low effluent concentrations. The pH and specific conductance must be measured at the beginning of the test in the control and the high, medium, and low effluent concentrations.

The temperature should be monitored continuously during the test with a recording thermometer submerged in one test chamber. Chemical analysis should be performed on the dilution water to indicate that it meets the recommended specifications.

Methods used for analysis of water quality must be those specified in Reference 21, 24, and 31. Residual chlorine can be measured to 3 ug/l using a modified amperometric method (Ref. 27). The concentration of un-ionized ammonia can be calculated from the concentration of total ammonia, pH, and temperature according to Table 8.

Range-finding Test. Unless the approximate toxicity of the effluent is already known, it is usually desirable to conduct a range-finding test to determine the concentrations that should be used in the definitive test.

TABLE 8. PERCENTAGE OF AMMONIA THAT IS UN-IONIZED IN DISTILLED WATER AT DIFFERENT TEMPERATURES AND pH'S (Ref. 30)

Temperature C	pH								
	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0
7	0.01	0.05	0.15	0.46	1.43	4.44	12.8	31.7	59.5
12	0.02	0.07	0.22	0.68	2.13	6.44	17.9	40.8	68.5
17	0.03	0.10	0.32	1.00	3.08	9.14	24.1	50.2	76.1
22	0.04	0.14	0.43	1.43	4.39	12.7	31.3	59.2	82.1
27	0.06	0.21	0.65	2.03	6.15	17.2	39.6	67.4	86.8

Generally groups of five organisms are exposed to three to five widely spaced effluent concentrations and a control for 8 to 24 hours. The greater the similarity between the range-finding test and the definitive test, the more useful the results of the range-finding test will be.

Meaningful range-finding tests may often be difficult to conduct, because the characteristics of the effluent may vary significantly within short periods of time. However, many nonchlorinated effluents have an LC50 between 2 percent and 100 percent. If a range-finding test is to be conducted with the same grab sample of the effluent with which a definitive test is to be conducted, the range-finding test can last 8 hours at the most.

Definitive Test. For the determination of an LC50 or an EC50, a control and at least five concentrations of effluent in a geometric series should be used. More treatments are desirable to insure the acceptability of the test and to provide additional data for various lengths of exposure. A definitive test must meet both of the following criteria so that the LC50 can be calculated with reasonable accuracy:

(1) Except for the controls, the concentration of toxicant in each treatment must be at least 50 percent of the next higher one.

(2) One treatment other than the control must have killed or affected less than 35 percent of the organisms exposed to it, and one treatment must have killed or affected more than 65 percent of the organisms. This requirement does not apply if 100 percent effluent does not kill or affect more than 65 percent of the organisms exposed to it.

If an LC or EC near the extremes of toxicity is to be calculated, such as an LC10 or an EC90, at least one treatment must have killed or affected a percentage of test organisms, other than 0 percent and 100 percent, near the percentage for which the LC or EC is to be calculated. This requirement might be met in a test to determine an LC50 or an EC50, but special tests with appropriate toxicant concentrations will often be necessary.

Other ways of providing information concerning the extremes of toxicity are to report the highest concentration of toxicant that actually killed or affected no greater a percentage of the test organisms than did the control treatment in the toxicity test or to report the lowest concentration of toxicant that actually killed or affected all of the test organisms exposed to it. These alternatives are normally more informative than reporting a result such as an LC2 or an EC98 unless several partial kills or effects are obtained close to 2 percent or 98 percent.

Calculations. Graphical interpolation provides the simplest means of obtaining an estimated LC50 value. The data are plotted on semilogarithmic coordinate paper with concentrations on the logarithmic axis and the percentages of affected fish on the arithmetic axis. A straight line is drawn between two points representing death in concentrations that were lethal to more than half and less than half of the animals. The concentration at which the line crosses the 50 percent death line is the LC50 value. If 50 percent of the animals are not killed by the highest concentration, the percent killed should be reported. Graphical interpolation does not, however, provide any confidence limits for the LC50 value.

For the purpose of determining the relative toxicity of industrial effluents, the graphical interpolation procedure is sufficient. However, if desirable, there are a variety of methods that can be used to calculate an LC50 and EC50 or any other LC or EC values (Ref. 24-25) and confidence limits. The most widely used are the probit, logit, moving average, and Litchfield-Wilcoxon (Ref. 27) methods. For each set of data, the LC50 or EC50 value and its 95 percent confidence limits can be calculated using the initial volume percent of the effluent in the test solutions. The percentage of test animals that are in the control treatment must not be used in the calculation of the results. The raw data should always be available so that the statistical methods can be applied if desired.

Reporting. Any deviation from these methods should be reported as well as the following specific information:

1. The chemical characteristics of the dilution water;

2. Detailed information about the test organisms, including scientific name, standard length, weight, age, life stage, source, history, observed diseases, treatments, and acclimation procedure used;

3. A description of the experimental design and the test chambers, the way the test was begun, the number of organisms per treatment, the loading, the lighting;

4. Definition of the criterion used to determine the effect and a summary of general observations on other effects or symptoms;

5. Percentage of organisms that died or showed the effect in the control treatment;

6. For Daphnia the 24- and 48-hour and for the fathead minnow the 24-, 48-, and 96-hour LC50 or EC50 values and the method(s) used to determine them. If 100 percent effluent does not kill or affect more than 50 percent of the test organisms, report the percentage of organisms killed or affected by various concentrations of the effluent.

7. Methods used for, and the results of, all chemical analyses of water quality and toxicant concentration, including validation studies and reagent blanks.

8. The average and range of the acclimation temperature and the test temperature.

3.4.3 Bioassay With Unicellular Marine Algae

3.4.3.1 Introduction

The community of unicellular algae is a very important constituent of marine ecosystems. It is comprised of a variety of species that have different growth rates, photosynthetic rates, nutrient requirements, and other functions that regulate species composition and diversity in the community in relation to environmental parameters. The algal community, through photosynthesis, produces most of the food and oxygen used in the marine ecosystem.

It is well known that algal species and communities are sensitive to environmental changes. Species may be inhibited or stimulated by pollutants. In a community, a pollutant may affect some species but not others, thereby causing changes in species diversity and composition. This can be followed by changes in composition of the animal community and altered routes of flow of energy and materials. Often, the altered ecosystem is undesirable from the human standpoint.

It is necessary, therefore, that a bioassay program designed to study effects of suspected pollutants include adequate research on unicellular algae.

3.4.3.2 Method Description

Facilities for Growth of Algae. Experimental and stock algal cultures must be maintained under carefully controlled environmental conditions. The easiest way to do this is to keep them in a walk-in controlled-environment chamber with shelves that allow for illumination of cultures from above by "cool white" fluorescent tubes. Light intensity should be approximately 4000 lux and temperature should be 20 $\pm$ 2 C. The lighting cycle should be 14 hours of light followed by 10 hours of darkness.

Algal Species. Algal species vary in their responses to toxicants. For example, a material may be highly toxic to one species but not another, while a second material may have the opposite effect. It might be best if species indigenous to an industry's area were used, but, because of variation in species response, it is recommended that Skeletonema costatum be used for ranking purposes. This alga is available from the Culture Collection of Algae, Department of Botany, University of Texas, Austin, Texas 78712.

Culture Medium. Since the main purpose of industrial waste bioassays is the ranking of industries according to toxicity of wastes, it is recommended that a single, artificial seawater salt mix be used. Although fortified natural seawater is a good medium for algal testing, geographic variation in composition may affect growth and thus affect ranking. Also, undiluted waste will be tested and it will be necessary to add solid salt mix to it.

The amount of salt added to test media approximates that found in low-salinity estuarine areas for two reasons:

1. Presumably, toxic effluents will usually be at their highest concentrations there.

2. The effect of a toxicant may be greatest when algae are under low-salinity stress.

Toxicity Tests. The recommended species grow well at low salinity. Saline culture media are to be prepared by adding a commercial salt mix (Rila Products, Teaneck, NJ 07666) to effluent to a concentration of 10 parts per thousand (ppt). Add approximately 10 gm of Rila Salts, with gentle swirling, to each liter of undiluted effluent. When the salt has dissolved, check the salinity by means of an instrument such as an American Optical Co. refractometer fitted with a salinity scale. Because artificial sea salts are hygroscopic, it will probably be necessary to add more salt. Do so carefully until a salinity of 10 ppt is reached after all salt is dissolved.

Diluting fluid is artificial seawater of 10 ppt salinity prepared with glass-distilled or deionized water. This will also be used as growth medium for control cultures.

Nutrients must be added to all control, diluted, and undiluted media. To each liter of medium, add 15 ml of metal mix, 1.0 ml of minor salt mix and 0.5 ml of vitamin mix as described in Table 9. The final concentrations of these nutrients are approximately one-half those needed for maximum growth and are used here so that enhancement of growth by effluent may be detected. Chelating agents should not be added to the media.

Stock Culture Medium. Algal stock cultures are to be maintained in autoclaved artificial seawater medium of 10 ppt salinity prepared from glass-distilled or deionized water. Nutrient concentrations should be the same as above. Stock cultures are grown in 150 ml of medium in 500 ml Erlenmeyer flasks and transferred every 10-14 days. Stock cultures must be manipulated according to standard microbiological techniques to insure a minimum of contamination by bacteria.

Glassware. All glassware must be of high-grade borosilicate glass (Pyrex or Kimex). Great care must be taken during cleaning to insure that contaminants from previous tests are not present. Each piece of glassware is first soaked in detergent for several hours and then hand-brushed, rinsed thoroughly with glass-distilled or deionized water, and rinsed three times with pesticide grade acetone. It is then soaked overnight in 10 percent nitric acid, again rinsed throughly with glass-distilled or deionized water, and oven-dried. When not in use, glassware must be stored in relatively dust-free containers or else covered by aluminum foil.

3.4.3.3 Procedure

Preparation of Test Algae. The algal bioassay method described here is the simplest one available and is designed to yield a large amount of data in as short a time as possible. It is not as precise as other methods in which cell counts are made each day or where actual living biomass is estimated, but it should be very adequate for ranking purposes.

TABLE 9 : COMPOSITION OF MIXES TO BE ADDED TO ALGAL GROWTH MEDIA

Nutrient Mixes	amount/Liter
Metal Mix[a]	
$FeCl_3 \cdot 6H_2O$	0,480g
$MnCl_2 \cdot 4H_2O$	0,144g
$ZnSO_4 \cdot 7H_2O$	0,045g
$CuSO_4 \cdot 5H_2O$	0,197mg
$CoCl_2 \cdot 6H_2O$	0,405mg
H_3BO_3	0,140g
Glass-distilled or deionized water	1 liter
Vitamin Mix[b]	
Thiamin hydrochloride	10 mg
Biotin	0,01mg
B_{12}	0,10mg
Glass-distilled or deionized water	100ml
Minor Salt Mix[c]	
K_3PO_4	1,0g
$NaNO_3$	10,0g
$Na_2SiO_3 \cdot 9H_2O$	20,0g
Glass-distilled or deionized water	1 liter

(a) Add 15 ml/l of test solution.

(b) Add 0.1 ml/l of test solution.

(c) Add 1,0 ml/l of test solution.

One week before a test is to begin, start an algal culture by adding, sterilely, 1.0 ml of a well-grown culture to 40 ml of 10 ppt artificial seawater with nutrients. Allow the algae to grow while being shaken under 4000 lux illumination (approximately) from "cool white" fluorescent tubes on a 14-hours light, 10-hours darkness cycle. On the day the test is set up, dilute this culture in sterile tubes with sterile artificial seawater to an optical density of 0.100 at 535 μm. A Fisher Electrophotometer II[7] (Fisher Scientific Co.) is convenient to use here because it accepts calibrated test tubes of 25 x 200 mm size (Bellco Glass Co., Vineland, NJ 08360). The calibration procedure for optically matching the tubes must be done in the testing laboratory.

Range-finding Tests. Range-finding tests are needed in bioassays to determine the concentrations of effluents to be used in definitive tests. Dilute the effluent that was taken to 10 ppt salinity with 10 ppt artificial seawater that contains nutrients to 0.01, 0.1, 1.0, and 10.0 percent. Filter-sterilize the dilutions by passing them through a sterile membrane filter such as the 0.22μ porosity filter manufactured by the Millipore Filter Corp., Bedford, MA 01730. At the same time, filter artificial seawater growth medium to be used for control cultures and undiluted effluent in a similar fashion.

Then add filter-sterilized medium aseptically to a sterile automatic pipette (such as Ace Glass, Inc. No. 8004-A fitted with No. 8004-36 volumetric bulb) and 25 ml added to each of three autoclaved 25 x 200 mm calibrated test tubes fitted with microbiological closures. This will yield 18 test culture tubes with three tubes each for control and 0.01, 0.1, 1.0, 10.0, and 100 percent waste. Add, aseptically, 0.5 ml of the cell suspension of 0.100 absorbance. Place the tubes on a shaker at approximately 60 excursions per minute. Model G2 Gyratory shaker fitted with No. AG2-TA75 platform (New Brunswick Scientific Co., New Brunswick, CN 08903) works well.

Read the absorbance of each tube against filtered medium on the fifth day after inoculation. Calculate the average absorbance for each group and the percentage growth in waste media as compared to the control. If growth in the lowest concentration is strongly inhibited, repeat the test with more dilute media. If inhibition of growth is found, estimate the concentrations, as percentage of controls, that will inhibit growth by approximately 85 and 15 percent.

Definitive Tests. For determination of the EC50, at least one control group and five concentration groups must be used. The five concentrations must be in a geometric series and include concentrations that inhibit growth by approximately 65 and 35 percent.

If growth stimulation occurred in the range-finding tests, use five concentrations in a geometric series between a concentration without effect and 100 percent waste.

The definitive tests are set up in the same way as range-finding tests except for different concentrations of effluent. Determine absorbance of the cultures every day between days 3 and 12. Plot the average absorbance for each day on graph paper and examine the shape of the curve. Some toxicants inhibit growth in the early stages of a test and it is necessary to be careful in interpretation of data.

Use the expression

$$\mu = \frac{\ln(X_2/X_1)}{t_2 - t_1}$$

where μ = specific growth rate

X_1 = absorbance on day t_1

X_2 = absorbance on day t_2

to calculate the specific growth rate between any days.

Estimate final biomass on the 12th day by weighing an aliquot of each culture. To do this, pass 200 ml of glass-distilled or deionized water through a 0.45u porosity membrane filter (Millipore). Dry the filter in an oven for 4-6 hours at 90 C. The filter may be dried on a sheet of paper that contains an identifying number. Cool the filter to room temperature in a desiccator and weigh it to the nearest one-tenth of a milligram.

Pass a suitable measured aliquot, usually 10 ml, of each culture through the filter under a vacuum of 0.5 atm. Do not use a greater vacuum because cells may break. Quickly, wash the cells with 5 ml of glass-distilled or deionized water to remove salts. Dry the filter with its attached algae for 4-6 hours at 90 C, cool to room temperature in a desiccator, and weigh to the nearest one-tenth of a milligram. Subtract the weight of the filter from the weight of the combined filter and algae and express algal weight to the nearest milligram.

Do the above for all tubes, calculate the average for each group, and express it as percentage of the control group.

3.4.3.4. Results

Calculation of the EC50. The EC50 is the concentration at which growth was 50 percent of the control. It may be estimated by interpolation by plotting the data on semilogarithmic coordinate paper with concentrations on the logarithmic axis and percentage growth on the arithmetic axis. To do so, draw a straight line between the two points on either side of the 50 percent growth value. The concentration at which the line crosses the 50 percent growth line is the EC50 value.

Stimulation. If growth was stimulated by the effluent, report the percentage stimulation at each concentration.

Report. In the final report, the following specific information should be included along with the general information provided for all bioassays:

1. The EC50 at 12 days and other days of importance to be decided upon by the shape of the growth curve.
2. The specific growth rate between days 3 and 12 and any other period that should be reported depending upon the shape of the growth curve.

3.4.4 Static Bioassays With Marine Animals (References 36-38)

3.4.4.1 Introduction

The method recommended for static bioassays on marine animals is the simplest, least expensive one available, and is designed to give a large amount of data in the shortest possible time. It incorporates methods given in Reference 36 and Reference 16. Juvenile sheepshead minnows (Cyprinodon variegatus) and adult grass shrimp (Palaemonetes pugio or P. vulgaris) are the test animals. These species adapt easily to a wide range of salinity and temperature in static bioassays and several phases of the life cycles can be studied.

The main objections to the method are that receiving water characteristics are not closely simulated and the test organisms may not be representative of the most sensitive species in a given geographical area. However, the method should be satisfactory for the purpose of ranking industries according to toxicity of their effluents.

3.9.9.8 Method Description

Facilities. Before tests are done, it is necessary to acclimate and observe the animals for several days. This requires tanks or live cars for holding in natural flowing seawater and tanks for acclimation. It is absolutely necessary that temperature be controlled in the holding and acclimation tanks and during the bioassay. The tanks must be constructed of materials that do not leach into, or sorb toxicants from, the water. Glass, #316 stainless steel, or perfluorocarbon plastics should be used whenever possible. Also, holding tanks should be constructed of plywood coated with fiberglass resin. Rubber, copper, brass, galvanized metal, or lead may not be used in any part of the test. Holding and acclimation tanks must be in a well-ventilated area that is free of fumes. They must be equipped for aeration, and the air, introduced via an air stone or glass tube, must be taken from a well-ventilated fume-free area. It may be pumped by an oilless rotary or piston type air compressor.

During holding, acclimation, and testing, the animals must not be disturbed unnecessarily, either by excessive handling or excessive movement around the tanks. When they must be handled, it should be as gently, carefully, and quickly as possible.

Species. Species to be used are juvenile sheepshead minnows (Cyprinodon variegatus) and adult grass shrimp (Palaemonetes pugio or P. vulgaris). Test shrimp may be collected from wild populations in relatively unpolluted areas, purchased from commercial suppliers, or cultured in the laboratory. Whatever the source, all animals used in testing should be healthy and as uniform in size as possible.

Juvenile sheepshead minnows are to be obtained from laboratory populations according to the method of Schimmel et al. (Ref. 17).

Test Containers. The static bioassays are to be done in 19.6-liter (5-gallon), widemouth, soft-glass bottles that contain 13 liters of test medium or in 30 x 60 x 30 cm deep, all-glass test chambers. If the rectangular test chambers are used, their sides should be bonded with clear silicon adhesive. As little adhesive as possible should be in contact with the water, and extra beads of adhesive should be on the outside of the containers rather than on the inside.

The test containers must be cleaned scrupulously before use. New containers must be washed with detergent, rinsed with tap water, then

pesticide-free acetone, and again with tap water. They must then be soaked for at least six hours in 10 percent hydrochloric acid, and rinsed with glass-distilled or deionized water. Between tests, the vessels must be treated in the same way, except they must be soaked for 2-4 hours in a solution of 200 mg hypochlorite/liter of glass-distilled or deionized water. This solution can be made by mixing six volumes of household chlorine bleach with 94 volumes of water. Never use hypochlorite when acid is present because hazardous fumes may be generated.

Containers must be rinsed thoroughly after cleaning. Just before use in a test, they must be rinsed with artificial seawater (see Diluted Effluent below).

Test Medium. The test medium is prepared from liquid effluent by addition of a commercial artificial sea-salt mix (Rila Products, Teaneck, NJ 07666). Salinity is 10 ppt because it is probable that industrial effluent will be in highest concentration in low-salinity water in estuaries.

Undiluted Effluent. Check the salinity of the effluent and add an appropriate amount of Rila Salts to each liter to yield a salinity of 10 ppt as determined by an American Optical Co. refractometer, or its equivalent, fitted with a salinity scale. Gently swirl or mix the effluent while adding salt.

Diluted Effluent. Prepare a Rila Salt solution of 10 ppt in glass-distilled or deionized water. Use this artificial seawater to dilute the salt-containing medium prepared with undiluted effluent. Dilutions will be prepared as percentages of undiluted medium.

Acclimation. Test the animals at 20 ± 2 C. If they are collected at another temperature (the fishes will be raised at 22 C), they should not be subjected to more than a 3 C change in temperature in any 1-hour period or to more than a 5 ppt change in salinity (the fishes will be raised at 10 ppt) in any 24-hour period. Avoid crowding during acclimation. Feed the animals a commercial flake food (Longlife Aquarium Products, Harrison, NJ 07029) once a day. Clean the acclimation tanks after each feeding.

The acclimation water is 100 percent dilution water. The animals should show no signs of stress, such as discoloration, altered behavior, or disease and must be kept for at least 3 days in acclimation tanks.

Remove dead and abnormal animals as soon as they are noticed. If more than 5 percent of a group dies in the 48 hours previous to testing, discard the whole group.

Treatment of Effluent Samples. Before testing, effluent samples must not be aerated or altered in any way. They should be covered at all times and never agitated. However, they may be swirled gently before a test to suspend particles or to aid in solution of Mix Salts. Initiate tests as soon as possible after the sample is taken and after range-finding tests are completed. Store the samples at 20 ± 2 C.

1.4.4.3 Procedure

Range-finding Tests. Because toxicity of an effluent probably will not be estimated, it will be necessary to run range-finding tests before definitive EC50 values can be calculated. Range-finding tests must be done in two ways, one with and one without aeration. In the test with aeration, introduce clean air at the rate of 100 ± 10 bubbles/minute from a glass tube of one mm diameter.

Use effluent concentrations of 0.01, 0.1, 1.0, 10.0 and 100 percent. If more than 50 percent of the animals die at 0.01 percent effluent, conduct a new range-finding test at lower concentrations such as 0.0001 and 0.001 percent. Conduct a control test of 100 percent dilution water at the same time. The pH of the test media and controls must be taken before and after the test.

Carefully place five animals in test containers at a weight not exceeding 0.8 gm/liter of medium. Determine weights by weighing representative members of the populations to be used before acclimation. Individuals that are to be used in a test must not be weighed or measured after acclimation has begun. Watch the animals for death or stress for 24 hours. In some cases, an 8-hour observation period may be sufficient but it should be recorded. Remove dead animals as soon as possible after death.

Definitive Tests. Concentrations of test effluent for definitive tests that yield LC50 or EC50 values will be determined from results of the range-finding tests. In the definitive tests, use a control and six concentrations of effluent in a geometric series with the concentration in each treatment at least 50 percent that of the next higher one. One

treatment must kill more than 05 percent of the animals and one treatment must kill less than 35 percent.

Do not feed animals during the definitive tests.

The bioassay exposures consist of two separate 96-hour bioassays, one with aeration and one without. This is because some effluents can be expected to have high BOD. Aeration is to be identical to that described in the range-finding test. Determine oxygen content of the water at 24-hour intervals by polarigraphic methods, and record pH and temperature for the same periods.

Observe the animals frequently throughout the 96 hours and record the number of dead animals for each 24-hour period. An animal should be considered dead if it does not respond to gentle prodding.

For shrimp, it may be necessary to calculate an EC50 value (effective concentration) rather than a LC50 (lethal concentration). This is because shrimp may not die from toxicants but may be immobilized and unable to move anything but the appendages. Loss of equilibrium may be another criterion for effect on shrimp.

For both shrimp and fish, report such things as erratic swimming, loss of reflex, discoloration, behavioral changes, excessive mucous production, hyperventilation, opaque eyes, curved spine, hemorrhaging, molting, and cannibalism.

3.6.4.4 Results

Calculation of LC50 or EC50. An LC50 is a concentration at which 50 percent of the experimental animals died and an EC50 is a concentration at which 50 percent of the experimental animals were affected. Either may be an interpolated value based on percentage of animals dying or affected at two concentrations. To estimate the EC50 or LC50, plot the data on semi-logarithmic coordinate paper with concentrations on the logarithmic axis and percentages of affected animals on the arithmetic axis. Draw a straight line between two points representing death or effect in concentrations that were lethal to or effective against more than half and less than half of the organisms. The concentration at which the line crosses the 50 percent mortality or effect line is the LC50 or EC50 value. If 50 percent of the animals were not affected by the highest concentration, the percent affected should be reported.

In some cases, only the 96-hr EC50 can be calculated because of color or suspended material that hide the animals in the waste.

Report. Any deviation from the above method should be reported. All reports should also include:

1. Weight of animals in each container.
2. Definitions of the criteria used to determine the effect, and a summary of general observations on other effects or symptoms.
3. Percentage of control organisms that died or appeared abnormal.
4. The 24-, 48-, and 96-hour LC50 or EC50.
5. Results of all pH, temperature, and oxygen measurements.

3.4.5 Stress Ethylene Plant Response (References 39,40)

This test is based on the well-known plant response to environmental stress: release of elevated levels of ethylene. Under normal conditions plants produce low levels of ethylene. The test is designed to expose plants to various levels of gaseous effluents under controlled conditions. The ethylene released during a set time period is then measured by gas chromatography to determine toxicity of the effluent.

3.4.5.1 Method Description

Materials. The recommended plant for this test is 6-week-old soybean, Glycine max (Dare variety). Other plants could be substituted, but all tests should be performed using one species, as plants vary in sensitivity to stress. Soybean is known to be very sensitive and is readily available in most areas. Optimum age of plants used for the test is also somewhat variable, but all plants used should be of the same age. Very young plants and, especially, senescent plants should not be used. Plants should be selected for uniformity of size and number of leaves. Great care should be taken during growth and exposure to insure that plants are not injured or stressed except by the feedstock or waste stream.

Greenhouse or growth chamber facilities with controlled temperature and lighting are necessary for growing the plants. It is suggested that the plants be grown under supplemental lighting to give a 16-hour day and under maximum day/night temperatures of 26 ± 3 C and 18 ± 3 C, respectively. Greenhouse exposure chambers equipped with an overhead light bank and an air-handling system, similar to those described in Reference 40 and a dark room at 26 C are also needed.

Procedure. The procedure used in this bioassay is a modification of that used in Reference 39. Because of limitations in gas sample size, Level 1 testing will be done using a static rather than a flow-through exposure. In addition, at this time the stress ethylene bioassay is under revision; therefore, what follows may be altered in the future. Plants are exposed to various concentrations of gaseous effluent for 3 hours in the greenhouse exposure chambers. Control plants must also be treated to determine normal ethylene levels. Immediately following exposure, each plant plus its pot are enclosed in a bag of known volume and incubated for 22 hours in a dark room at 26 C. The duration of ethylene release is usually short and increases with higher pollutant concentration.

Ethylene samples are taken from the bag by inserting a syringe needle, and the concentration is measured using a gas chromatograph equipped with a flame ionization detector. The presence of ethylene is confirmed by co-chromatography with standard ethylene. A variety of chromatographic conditions can be used for measuring ethylene. Analysis should be done as soon as possible after incubation as some ethylene will diffuse through the bag with time. The range for normal ethylene production in Glycine max is 25-40 nl ethylene/l air as reported in Reference 39. As most work has been done on the effects of known pollutants such as ozone, it is difficult to predict the levels of ethylene released in response to exposure to a grab sample. The exposure to 0.4 µl/l and 0.8 µl/l ozone causes release of 250 and 600 nl/l ethylene, respectively (Ref. 39).

It should be noted that very high levels of pollutant may cause death of plant tissue. In this case, little ethylene will be released as only wounded, living plant tissue produces ethylene. Plants should be inspected for such damage, and it should be reported if observed.

For this test, at least three levels of the sample and a control should be run. The sample gas should be pure (undiluted) and in dilutions of 0.5 and 0.25. If adequate gas sample is not available, the test must be run on more diluted samples. Four to ten plants should be exposed to each experimental condition and the control.

3.4.3.2 Results

Results from varying concentrations of gas exposure are plotted on semi-log paper and fitted by the least squares method to the following formula:

$$\ln C = \ln(A) + BX,$$

C = total ethylene concentration (nanoliters/liter),

A = amount of ethylene produced by a non-stressed plant,

B = measure of increase in stress-induced ethylene produced as a function of pollutant concentration,

X = pollutant concentration.

This formula takes into account the low levels of ethylene produced by plants under normal circumstances, and the use of natural logarithms stabilizes the variance of ethylene measurements at higher concentrations. In addition to reporting the slope parameter, (B), and its 95 percent confidence limit, specific information about the plants used and their growth conditions should be supplied for each sample.

3.4.3.3 Discussion

Release of ethylene is a well documented plant response to various kinds of stress. Advantages of this test include reliability, sensitivity, speed and economy. Disadvantages center on the extreme care needed in handling the plants to prevent any physical damage or stress which would confuse the test results.

3.4.6 Soil Microcosm Test (References 41-49)

3.4.6.1 Introduction

The Level 1 testing yields a rapid, yes-no solution to assessment queries. It quantifies the relationship between contaminant dose and transport, accumulation, and short-term effects. Since terrestrial accumulation sites and remineralization processes are predominantly within soil, intact soil microcosms excised from representative target systems are used as test units.

Techniques and procedures following have resulted from several individual experiments exploring the utility of microcosms as test systems for assessing potentially hazardous materials. These procedures have been tested using a variety of toxicants and have been compared to field derived data. (Ref. 41-44)

In order to implement an adequate, efficient assessment, some background data are needed. First, what is the mode of entry into the target ecosystems? If discernable, what is the frequency of discharge? Second, what are the chemical characteristics of the substance? Third, what information is available on direct toxicity to laboratory personnel? Fourth, what are the target ecosystems?

The immediate questions which must be addressed in assessing potentially hazardous substances are: (1) Where does it go? and (2) What and how much alternation of ecosystem processes and population occurs?

Initially, we must discern if there is significant transport and whether significant, short-term effects can be shown. These questions call for a rapid, highly accurate, binary (yes/no) experimental design. The key to answering this question is establishing a dose-transport and dose-effect relationship for the specific substance of interest. Substances should be tested on those ecosystem types (northern hardwood forest, pasture or desert, e.g.) which will be the likely recipient.

The soil microcosm test results in specific answers to five questions. These are: (1) Is the substance (or transformation or degradation product) mobile? (2) Is the primary mode of soil export gaseous, dissolved, or particulate? (3) Is nutrient cycling disruption indicated? (4) Are soil biota affected? (5) Is mobility, export mode, nutrient cycling disruption, or population effecting a function of dose?

The soil microcosm test has been shown rapid and accurate in answering these questions compared to total system microcosm and field studies (Ref. 42-45). This allows the user to: (1) Determine dose ranges for further testing; (2) discern whether there is reason for further terrestrial testing (substance not highly mobile) or whether aquatic studies are needed (substance highly mobile); and (3) rank substances being tested for priorities of Phase 2 and 3 testing.

3.4.6.2 Method Description

Obtain soil cores 5 cm diameter x 5 cm depth from representative terrestrial ecosystems. Remove above ground higher plant tissue.

Encase the cores in 1 mil thick teflon within 2 mil thick shrinkable polyvinyl chloride and gently heat shrink until a tight bond with the core (minimum boundary flow) is achieved. Leave enough lining above the soil surface to use gaseous export traps if necessary. Mount on glass funnels in test tubes. Cover sides with opaque wrapping to negate abnormal algae growth. Place in environmental chamber under as near the field conditions as possible (Figure 4).

Equilibrate 3 weeks, if possible. Leach with rainwater or reconstituted water (known water chemistry) 2-3 times (enough to obtain 20-30 ml/date during equilibration). Determine Ca and dissolved organic carbon (DOC) concentrations in these samples. If possible, use alkali traps to determine daily CO_2 efflux 3-10 days during equilibration. Use these data to discard dissimilar replicate soil cores and establish behavior of individual replicates.

Experimental design is preferably a randomized complete block and, if possible, factorial treatment arrangement of dosages with a minimum of three cores per dose per terrestrial ecosystem tested. Randomized incomplete block designs can be used to simultaneously test a large number of contaminants. Dosages of a wide range should be used for this phase to maximize the clarity of dose dependent observations.

Add the test contaminant to the surface of the cores in a carrier, such as soil (taken from replicate cores to those used as experimental units). Dosages might be 0, 10, 100, 1000 ppm, for example, based on core weights. Total amendment to all cores should be equivalent, so that, for example, a control replicate would receive carrier (such as soil)

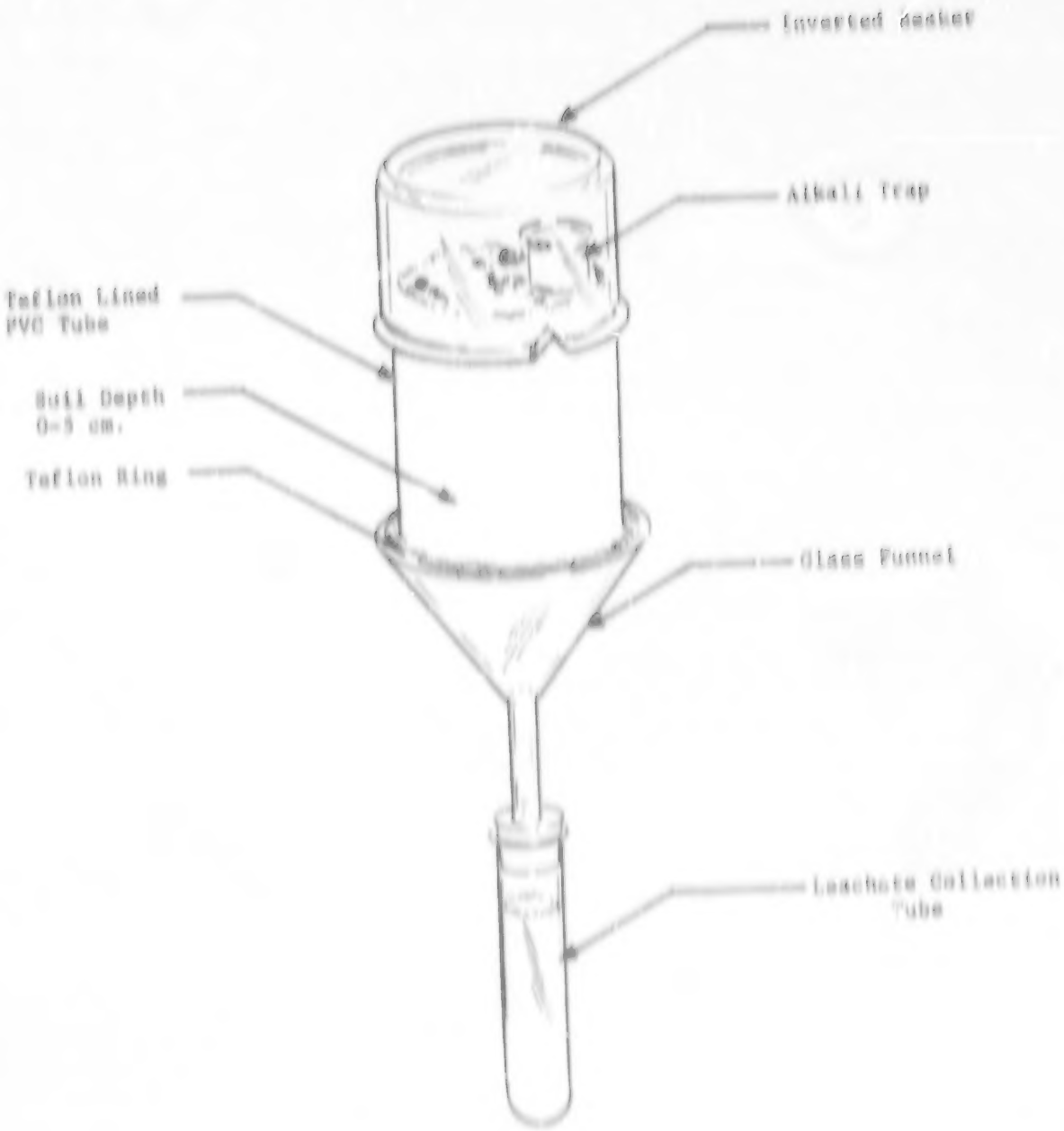

FIGURE 4: DIAGRAM OF A SOIL MICROCOSM UNIT

equivalent to the carrier plus contaminant received by any treatment dose. The following are weights of carrier and contaminant added to soil cores approximately 150 g each unit weight, 150 g air dry weight.

Dosage (ppm)	Carrier (mg)	Contaminant (mg)
0	150	0
10	178.2	1.8
100	162	18
1000	0	150

Contaminant and carrier should be mixed, and the particulate ground to 1 mm particle size. Deposition of treatments onto cores should be as even across the surface as possible.

Set gas traps to monitor CO_2 efflux. Standard alkali traps, such as 0.2 M KOH can be used for CO_2 recovery. Collect traps and titrate with 0.1 M HCl at 24, 48, or 72 hour intervals. The more frequent the measurements, the more complete the data analysis can be; however, diurnal rhythms make daily observation the minimum useful time period.

Traps to absorb volatile contaminants may be used to discern the existence and relative importance of gaseous export of the contaminant. TENAX® absorbs most organics quantitatively and can be eluted to recover them for detection.

After a week, add sufficient rainwater or reconstituted water (known water chemistry) to collect approximately 20 ml leachate per core. Analyse Ca and DOC concentration. Determine contaminant concentration using standard chemical techniques. On days 14 and 21 repeat leaching.

Perform intact extraction technique for pools of nutrients left within the core to estimate mass balances for microcosm units. (Ref. 46) This technique is the addition of 200 ml of 1.0 M HCl or $NaHCO_3$ to cores and measurement of Ca, DOC and the contaminant in the leachate respectively.

Biotic analyses should also be conducted. Core samples 1 cm in diameter should be removed from the soil microcosms using a cork borer. The hole should be filled by a glass rod of the appropriate size if extraction for contaminant and nutrients is to be performed.

Biotic analysis should use the ATP assay, which would allow relative microbial pools to be compared across treatment levels. The procedure for ATP analysis is to add 1 g soil (wet weight) to 6 ml 7.4 pH Tris buffer

with 0.06 g ethylene diamine tetraacetic acid (EDTA). Vortex briefly Add 3 ml chloroform. Vortex again. Sonify in ice water 2-3 minutes. Centrifuge (preferably at low temperature) at 1000 G for 2-10 minutes. Transfer buffer to new tube. Add 3 ml CCl_4. Recentrifuge briefly. Sample buffer phase. Assay at 340 nm using standard hexokinase reaction (commercial kit available from Calbiochem Corporation (Ref. 47, 48).

Divide cores into 1 cm depths. Within each depth measure the amount of contaminant by radioisotope techniques or stable chemistry. This is an optional step, useful if the distribution of the contaminant is to be estimated.

3.4.6.3 Results

Two types of data will be produced: monitoring data and harvest data. Monitoring and harvest data will be available on nutrients processes as well as on contaminant transport and fate.

Monitoring Results. Calculate the total export of Ca, DOC and contaminant for each microcosm by data using concentrations detected and volumes of leachate collected. Calculate mean export (with standard error) by treatment dose for each contaminant. Plot these data as cumulative export as a function of time. (See Figure 5). CO_2 efflux or other gaseous export data can be similarly summarized and presented. Statistical comparison can be made by coveriance to determine the effect of treatment on export of nutrients and contaminant. Previous studies show that often CO_2 efflux and nutrient export increases as a function of dose. Transport of the contaminant is usually greater with increasing dose.

Harvest Results. Calculate the extractable Ca, DOC, or contaminant based on concentrations of ions measured multiplied by extractant volumes. Calculate means (with standard errors) across replicates for each treatment dose. Use standard analysis of variance and Duncan's Range Tests to determine differences due to treatment. The following is an example of data which would result from extraction procedures at harvest.

Contaminant Dosage (ppm)	Extractable Ca (mg/microcosm)
0	230 ± 23
10	260 ± 10
100	106 ± 20
1000	83 ± 50

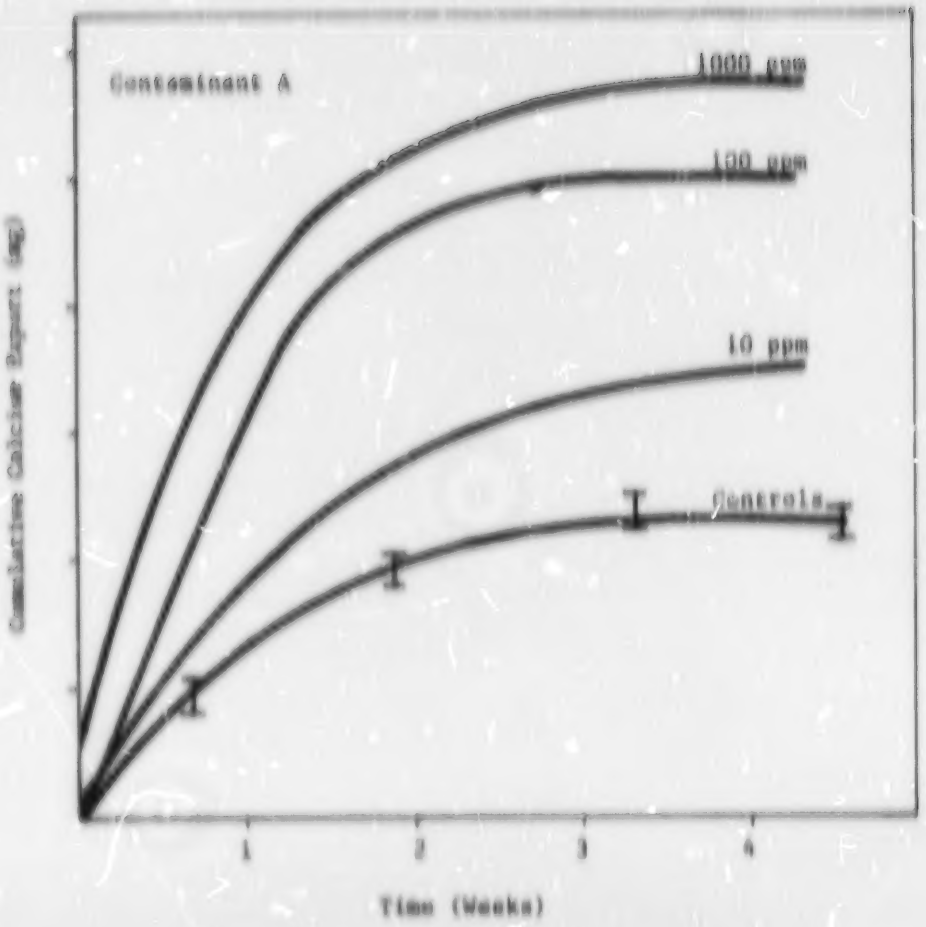

FIGURE 5. SAMPLE PLOT OF CUMULATIVE CALCIUM LOSS AS A FUNCTION OF TIME FOR 4 DOSAGE LEVELS OF A CONTAMINANT.

Biotic data can be summarized as ATP per gram soil by 1 cm depth intervals within each dosage. ATP concentration may be increased or decreased by contaminant, depending upon specific microbial group impacted.

Reporting Data. Data summaries should include information on source of soil cores, dates taken from the field, whether equilibration was made before treatment, and conditions under which cores were maintained. An example data summary sheet is given in the Appendix.

3.4.6.4 Discussion (Ref. 45)

Replicability. In order to establish quality control of screening procedures, three criteria must be met. First, the parameters must be predictable through the experimental period. Usually, this requirement necessitates equilibration of the experimental units prior to treatment with test substance. Second, confidence limits must be established for the measured parameters. Unique microcosms for each substance, or each dose rate are inapplicable. Third, reproducibility of parameters between control microcosms between comparable experiments must be reasonable.

Replicability among experimental units within a treatment class has been calculated during experiments using intact, homogenized, and sand-soil substrates. We have found that replicability is dependent upon substrate type and upon the parameter estimated. For example, CO_2 efflux was replicable in all substrates, but DOC efflux varied greatly among homogenized and sand-soil replicates at each sampling date and through time. Similar results were obtained for nine nutrients. Results from grass microcosms established using these substrates were similar. We conclude that replicability of intact soil microcosms is greater than other substrates.

Reproducibility. Several comparable experiments have been conducted using the intact soil profile, total system microcosms, and field studies. Overlapping error terms for common parameters measured in control units of these experiments allow us to conclude reproducibility of the technique.

Accuracy. The criteria for determining microcosm result accuracy are dual. First, parameters indicative of ecosystem processes should be similar to those measured within comparable strata of the intact ecosystem. Second, the distribution and the transport rates of the test substance should mimic those which would occur in the source ecosystem. These

criteria are the most difficult of those needed to establish the utility of microcosms in screening potentially hazardous substances. Only limited data have been collected to address this issue. The comparisons below represent our findings to date.

Evidence was obtained from forest microcosm experiments. (Ref. 42) Using comparable data for cadmium, zinc, copper, and lead, microcosm data were compared to the forest ecosystem under stress from the heavy metal deposition. (Ref. 43) Enrichment ratios, ER, were computed by dividing the heavy metal concentration of the treated microcosm component by the same metal concentration of the control microcosm component. Greatest enrichment of metals occurred in litter strata. Cadmium had greater ER for soil than did other metals, although Pb was greatly enriched. Values compared to those near the heavy metal source, were similar to the sampled ecosystem component. (Ref. 42)

More conclusive proof of accuracy was the nonsignificant difference in field and microcosm data taken on similar dates, for parameters such as CO_2 efflux, calcium, and DOC.

Problems. Several problems remain in the evaluation of the utility of microcosms as screening tools to assess transport, fate and effects of potentially hazardous substances. Four problems seem important. These are: (1) the rate of ecosystem degradation cannot be calculated from loss rates of nutrient elements as yet, although in theory it is possible to do so; (2) the microcosms have not been tested using organic effluents, singly or in combination; (3) absolute accuracy of microcosm results has not been proven; and (4) reproducibility among microcosm studies conducted using intact profiles excised during different seasons has not been established.

Some information is needed to conduct adequate screening of potentially hazardous substances. The mode of entry of the substance into the system is needed. The direct toxicity of the substance to research workers is needed in order to practice safe laboratory experiments. The potentially targeted ecosystems should be identified if possible and used as sources of microcosm units. Lacking this information, forest or pasture systems should be used since more than 80 percent of the non-urban U.S. is one of these ecosystems.

5.5 REPORTING FORMAT

The report of the results of any bioassay should include the following:

(1) Name of the laboratory and investigator conducting the test,

(2) Dates of the test,

(3) Detailed description of the material tested including its source, date and time of collection, and any known information on its physical and chemical properties obtained from observation and on-site testing (e.g., color, turbidity, pH, and solubility).

(4) Detailed description of the testing procedure including information on the test organisms, materials, and apparatus used for the test, and on sample handling (e.g., dilution and solvent factors, mode of administering).

(5) Results of the tests including all preliminary tests on range finding or mode of administering plus all control tests performed. Qualitative as well as quantitative observations should be reported. Raw data in the form of a table or graph should be included along with final calculations such as percent inhibition at various effluent concentrations, EC50 or LC50, and maximum tolerated dose estimate plus statistical analysis where appropriate. For some tests the final form of the data will be a table or graph.

(6) Any other relevant information.

CHAPTER IV

APPROACH TO LEVELS 2 AND 3 BIOLOGICAL TESTING

4.1 INTRODUCTION (Reference 3)

Level 1 analysis provides a survey of effluent streams which permits ranking on a priority basis or distinguishing extremely hazardous streams from those which are less hazardous or innocuous. Level 1 sampling and analysis procedures are suitable for analyzing a variety of materials and should ensure a high probability of detecting potential environmental problems; however, the procedures will not necessarily provide information on specific substances.

With the information provided by Level 1 analysis, a more detailed, more quantitative analysis of the streams in order of their relative importance can be performed (Level 2). Level 2 sampling and analysis programs are directed towards a confirmation of Level 1 results as well as a more detailed characterization of the biological effects of the more toxic streams. They are not as broad as Level 1 in that resources are expended to improve information on streams of a critical nature. Additional sampling of other streams is deferred because Level 1 information has indicated a less significant level of environmental impact. In some cases, Level 2 may make use of the same procedures or modifications of the procedures used in Level 1. In many cases, more sophisticated techniques may be required. A major difference in the two levels is that at Level 2, greater attention is given to acquiring representative samples and to more replication of samples. Furthermore, Level 2 analysis will not be conducted via a prescribed series of tests. Each sample will require selection of appropriate techniques based on the information developed in Level 1 and the information requirements of the assessments. Level 2 analyses are the most critical of all three levels because they must provide a valid estimate of toxicity. It is equally important, however, that the analyses are conducted in an information effective manner because increasing specificity and/or accuracy result in cost escalations. Due to the multiplicity of analytical techniques required and the potential for unnecessarily high resource expenditures, the

Level 2 analyses should be managed by experienced analytical personnel working in well-equipped laboratories. Furthermore, the analyses must be conducted with full awareness of the information requirements of an environmental assessment program. Biological analysis as well as chemical and physical analysis should be performed at Level 2. Suggestions for possible Level 2 bioassays are included in this chapter.

Level 3 sampling and analysis is designed to monitor a limited number of selected compounds and to define accurately chronic sublethal effects of the selected compounds. *In vivo* monitoring should be incorporated, if possible. Since the analytical procedures are highly process and site specific, it is not possible to define their exact form. In general, analysis will be optimized to a specific set of stream conditions, and therefore, Level 3 tests will most probably not be as complex or expensive as Level 2 tests. It should be stressed that the Level 2 and 3 biological tests listed here are suggestions only. They are not recommended or required tests and should be considered only as examples of the *kind* of test appropriate to a particular level.

4.2 APPROACH TO HEALTH EFFECTS TESTING

Possible Level 2 bioassays include *in vitro* and *in vivo* tests to confirm possible toxicity or mutagenicity detected at Level 1. Any biotesting to be done at Levels 2 and 3 will be done through the Office of Health and Ecological Effects, Research Triangle Park. Suggested Level 3 tests include *in vivo* analyses for chronic toxicity, mutagenesis, carcinogenesis, teratogenesis, and metabolism. Such procedures provide more quantitative information on possible health risks.

4.3 APPROACH TO ECOLOGICAL EFFECTS TESTING

Examples of Level 2 bioassays include bioaccumulation and persistence studies to provide a more detailed analysis of the effluent streams. Possible Level 3 bioassays include tests of toxic substances removal by waste treatment facilities, mutagenicity, and ecosystems analysis.

One possible terrestrial ecological effect bioassay at Levels 2 and 3 is phytotoxicity of airborne substances. Toxicity is indicated by any foliar lesion produced by exposure to the test substance. Level 2 testing would be designed to establish relative toxicity using selected plant species. Level 3 would be designed to establish the threshold for plant

tory. At both levels only direct effects on plants are considered by this test. Not considered are such indirect effects as: (1) alteration by a toxicant of disease susceptibility, (2) accumulation of a toxicant on plant growth and yield. Possibly bioassays taking such factors into account could be developed in the future.

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APPENDIX

BIOASSAY RECORD SHEETS

GENERAL FORMAT FOR MICROBIAL TOXICITY OR MUTAGENICITY DATA

Test Condition	Sample Type	Assay Amount (ml/plate)	Revertants on Individual Plates				Average		Adjusted Average Revertants		Index of Relative Mutagenicity	
			Number 1	Number 2	Repeat 3	Repeat 4						
Reactivation	Positive control											
	Solvent control											
	Sample											
Induced activation	Positive Control											
S9	Solvent control											
	Sample											

VI-78 CELLULAR TOXICITY TESTING

Sample No._____
Date Rec'd_____
Description of Sample_____
_____
Date Tested_____
Date Report Out_____
Passage of Cells_____
Seeding Population of Cells_____
Incubation Time_____

EC_{50} VALUES
Cell Count_____
Viability_____
Viability Index_____
Protein_____
ATP_____
Other_____

TEST RESULTS

Tube No.	Conc. µg/ml or (ml/ml)	pH Initial	pH After Incub.	Cell No. as % of Control	Viable Cells	Viability Index	ATP	Protein

ALVEOLAR MACROPHAGE TOXICITY TESTING

Sample No._____
Date Rec'd_____
Description of Sample_____
_____
Date Tested_____
Date Report Out_____
No. Rabbits Used_____
Remarks About Rabbits_____
_____
Total No. Cells Recovered_____
Seeding Population of Cells_____

DIFFERENTIAL
Macrophages_____
Neutrophilis_____
Other_____
Incubation Time_____
EC_{50} Values_____
Cell Count_____
Viability_____
Viability Index_____
Protein_____
Other_____

TEST RESULTS

Tube No.	mg/ml or ml/ml	pH Initial	pH After Incub.	Cell No. as % of Control	Viable Cells	Viability Index	ATP*	Protein

*ATP/10^6 cells as % of Control

STRESS ETHYLENE PLANT RESPONSE RECORD SHEET

Sample No.: _____

Date Rec'd _____

Description of Sample _____

Date Tested _____

Date Report Out _____

Plant Material Used: _____

Age of Plants _____

Growth Conditions Light Cycle: _____

Light Intensity: _____

Day/Night Temp.: _____

Conc. of Gas Sample Used: _____

No. of Plants Tested/Conc.: _____

TEST RESULTS

Plant No.	Conc., Percent	Ethylene Release Nanoliters/liter

ALGAL BIOASSAY DATA SHEET

LABORATORY_____ DATE_____

INVESTIGATOR_____ CULTURE MEDIUM_____

TEST NO._____ pH OF MEDIUM_____

TEST SAMPLE SOURCE_____ TEMPERATURE_____

COLLECTION DATA (TIME-DATE)_____ ILLUMINATION_____

OTHER PERTINENT SAMPLE INFO._____

RESULTS

Sample Flask No.	Conc.	INCUBATION TIME-DAYS 3			5			7			(etc for 9,13,17, 21 Days)
		Cells/ml	Dry wt. mg/L	Other	Cells/ml	Dry wt. mg/L	Other	Cells/ml	Dry wt. mg/L	Other	

REMARKS

Maximum Specific Growth Rate:
Maximum Standing Crop:
EC 50(12 Day or other dyas of importance)

[redacted]

STATIC BIOASSAY RECORD SHEET

Series: _____ Municipality or Company: _____ Date: _____

Technician: _____ Starting hour: _____

Material being tested: _____

Source: _____

Source of dilution water: _____

Test species: _____ Temp. range: _____

No. individuals per percent waste: _____

	Start									Control	
Percent waste											
DO											
Temperature											
pH											
Specific conductance											
	24 hours										a Lc_{30}/EC_{30}
Number surviving											
% survival											
DO											
Temperature											
pH											
	48 hours										
Number surviving											
% survival											
DO											
Temperature											
pH											
	96 hours										
Number surviving											
% survival											
DO											
Temperature											
pH											

a Method used for calculating.

SAMPLE DATA SUMMARY FOR SOIL MICROCOSM TEST

Date of Report:_____ Investigator:_____

Contaminant:_____ Source:_____Date:_____

Microcosm design_____

Treatment levels (dosages)_____

Replications_____

Carrier_____

Microcosm Source_____Date Taken:_____

Equilibration Period_____

CO_2 Efflux ($\bar{X} \pm$ S.E.)_____mg/day

Ca Export ($\bar{X} \pm$ S.E.)_____ng/week

D.O.C. Export ($\bar{X} \pm$ S.E.)_____ng/week

Treatment Date_____

Monitoring Data:

CO_2 Efflux:

dose	$\bar{X} \pm$ S.E.(mg/day)	cumulative loss(mg)

Ca Export:

dose	$\bar{X} \pm$ S.E.(mg/week)	cumulative loss(mg)

DOC Export:

dose	$\bar{X} \pm$ S.E.(mg/week)	cumulative loss(mg)

SAMPLE DATA SUMMARY FOR SOIL MICROCOSM TEST
(Continued)

Monitoring Data:

Contaminant Export:

Detection method_____

dose | $\bar{X} \pm S.E.$ (ng/week) | cumulative loss (mg)

Harvest Data:

Extractable Ca: Extractant_____

dose | mg/g soil

Extractable D.O.C. Extractant_____

dose | mg/g soil

Extractable Contaminant: Extractant_____

dose | mg/g soil

Distribution of Contaminant:

depth (cm)	dose (mg/g soil)
1	
1-2	
2-3	
3-4	
4-5	

SAMPLE DATA SUMMARY FOR SOIL MICROCOSM TEST
(Continued)

ATP Concentration:

dose depth(cm)	mg/g soil
0-1	
1-2	
2-3	
3-4	
4-5	

Summary:

Contaminant ( ) increased/decreased/did not change monitored parameters, and increased/decreased/did not change harvest parameters.